Evaluierung des Zoonotischen Risikos von *Escherichia coli* Stämmen assoziiert mit Extraintestinalen Infektionen bei Menschen und Tieren. Charakterisierung Neuer Virulenzfaktoren von ExPEC

Evaluation of the Zoonotic Risk of *Escherichia coli* Strains involved in Extraintestinal Infections of Humans and Animals. Characterization of New Virulence Factors in ExPEC

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1. Summary

Avian pathogenic *Escherichia coli* (APEC) represent a subset of the so-called extraintestinal pathogenic *Escherichia coli* (ExPEC) pathotype that can cause various extraintestinal infections in humans and animals. APEC are the causative agent of localized colibacillosis or systemic infection in poultry. In this latter case, the syndrome starts as an infection of the upper respiratory tract and develops into a systemic infection. Generally, ExPEC are characterized by a broad variety of virulence-associated factors that may contribute to pathogenesis. Major virulence factors, however, that clearly define this pathotype, have not been identified. Instead, virulence-associated genes of ExPEC and thus also of APEC could be used in a mix-and-match-fashion. Both pathotypes could not be clearly distinguished by molecular epidemiology, and this suggested a hypothetical zoonotic risk caused by APEC.

Accordingly, the main scientific question of this study was to characterize common traits as well as differences of APEC and human ExPEC variants that could either support the possible zoonotic risk posed by these pathogenic *E. coli* strains or indicate factors involved in host specificity. Comparative genomic analysis of selected APEC and human ExPEC isolates of the same serotype indicated that these variants could not be clearly distinguished on the basis of (i) general phenotypes, (ii) phylogeny, (iii) the presence of typical ExPEC virulence genes, and (iv) the presence of pathoadaptive mutations. Allelic variations in genes coding for adhesins such as MatB and CsgA or their regulators MatA and CsgD have been observed, but further studies are required to analyze their impact on pathogenicity.

On this background, the second part of this thesis focused on the analysis of differences between human ExPEC and APEC isolates at the gene expression level. The analysis of gene expression of APEC and human ExPEC under growth conditions that mimick their hosts should answer the question whether these bacterial variants may express factors required for their host-specificity. The transcriptomes of APEC strain BEN374 and human ExPEC isolate IHE3034 were compared to decipher whether there

was a specific or common behavior of APEC and human ExPEC, in response to the different body temperatures of man (37°C) or poultry (41°C). Only a few genes were induced at 41 °C in each strain relative to growth at 37 °C. The group of down-regulated genes in both strains was markedly bigger and mainly included motility and chemotaxis genes. The results obtained from the transcriptome, genomic as well as phenotypic comparison of human ExPEC and APEC, supports the idea of a potential zoonotic risk of APEC and certain human ExPEC variants.

In the third part of the thesis, the focus was set on the characterization of Mat fimbriae, and their potential role during ExPEC infection. Comparison of the *mat* gene cluster in K-12 strain MG1655 and O18:K1 isolate IHE3034 led to the discovery of differences in (i) DNA sequence, (ii) the presence of transcriptional start and transcription factor binding sites as well as (iii) the structure of the *matA* upstream region that account for the different regulation of Mat fimbriae expression in these strains. A negative role of the H-NS protein on Mat fimbriae expression was also proven at 20 °C and 37 °C by real-time PCR. A major role of this fimbrial adhesin was demonstrated for biofilm formation, but a significant role of Mat fimbriae for APEC *in vivo* virulence could not yet be determined. Interestingly, the absence of either a functional *matA* gene or that of the structural genes *matBCDEF* independently resulted in upregulation of motility in *E. coli* strains MG1655 and IHE3034 by a so far unknown mechanism.

In conclusion, the results of this thesis indicate a considerable overlap between human and animal ExPEC strains in terms of genome content and phenotypes. It becomes more and more apparent that the presence of a common set of virulence-associated genes among ExPEC strains as well as similar virulence gene expression patterns and phylogenetic backgrounds indicate a significant zoonotic risk of avian-derived *E. coli* isolates. In addition, new virulence factors identified in human ExPEC may also play a role in the pathogenesis of avian ExPEC.

Zusammenfassung

Vogelpathogene *Escherichia coli* (APEC) sind eine Untergruppe der sogenannten extraintestinal pathogenen *Escherichia coli* (ExPEC), welche Infektionen außerhalb des Verdauungstraktes beim Menschen und vielen Tieren verursachen können. ExPEC sind durch eine Vielzahl Virulenz-assoziierter Faktoren charakterisiert, die zur Pathogenese beitragen können. Haupt-Virulenzfaktoren, die eine eindeutige Zuordnung zu diesem Pathotyp erlauben, wurden jedoch noch nicht identifiziert. Die Virulenz bei ExPEC und somit auch bei APEC scheint auf der kombinierten Expression von Virulenzfaktoren zu beruhen. Beide Pathotypen können daher nicht eindeutig aufgrund des Genomgehaltes sowie molekularer Epidemiologie voneinander unterschieden werden.

In der vorliegenden Arbeit sollten Gemeinsamkeiten sowie Unterschiede bei ausgewählten APEC- und humanen ExPEC-Isolaten des gleichen Serotyps untersucht werden, um nähere Hinweise auf ein Zoonoserisiko zu erhalten oder um Faktoren zu charakterisieren, die zur Wirtsspezifität beitragen können. Vergleichende Analysen des Genomgehaltes zeigten, dass diese Varianten nicht aufgrund (i) genereller Phänotypen, (ii) ihrer Phylogenie, (iii) der Anwesenheit typischer Virulenz-assoziierter Gene sowie (iv) pathoadaptiver Mutationen voneinander unterschieden werden können. Interessanterweise wurden bei manchen Isolaten Allelvariationen in Genen beobachtet, die für Adhäsine wie MatB und CsgA sowie für ihre Regulatoren (MatA und CsgD) kodieren. Ihre mögliche Bedeutung für die Virulenz muß jedoch weiter analysiert werden.

Im zweiten Abschnitt dieser Arbeit wurde untersucht, ob sich eng verwandte Vogel- und humane ExPEC-Isolate hinsichtlich ihrer Genexpression unterscheiden. Um zu untersuchen, ob die Körpertemperatur des Menschen (37 °C) oder von Geflügel (41 °C) einen unterschiedlichen Einfluß auf die bakterielle Genexpression hat und somit zur Wirtsspezifität beitragen kann, wurden die Transkriptome des APEC-Stammes BEN374 und des humanen ExPEC-Stammes IHE3034 nach Anzucht *in vitro* bei 37 °C bzw. 41 °C miteinander verglichen. Wachstum bei 41 °C führte nur bei wenigen Genen zu einer Induktion der Genexpression, wohingegen die Anzahl der reprimierten Gene bei dieser

Temperatur in beiden Stämmen deutlich höher war und vor allem auf eine reduzierte Beweglichkeit und Chemotaxis hindeutete. Die Ergebnisse von vergleichender Genomik, Transkriptomik und Phänotypisierung humaner ExPEC- und APEC-Stämme unterstützen somit die Annahme, dass es ein Zoonoserisiko zwischen manchen APEC- und humanen ExPEC-Isolaten gibt.

Im dritten Teil dieser Arbeit stand die Charakterisierung der Mat Fimbrien-Expression in *E. coli* sowie ihre Rolle bei der Infektion im Mittelpunkt. Der Vergleich der kodierenden *matABCDEF* Determinanten im *E. coli* K-12 Stamm MG1655 und im humanen ExPEC O18:K1 Isolat IHE3034 zeigte Unterschiede in (i) der jeweiligen Nukleotidsequenz, (ii) der Anwesenheit von Transkriptionsstartpunkten und Transkriptionsfaktor-Bindungsstellen sowie (iii) der Struktur der „Upstream“-Region des Genclusters auf, die zur unterschiedlichen Fimbrienexpression in beiden Stämmen beitragen können. Eine Repression der Mat Fimbrienexpression durch das H-NS Protein wurde nachgewiesen. Zudem wurde gezeigt, dass Mat Fimbrien signifikant zur Biofilmbildung beitragen, wohingegen ein Beitrag zur *in vivo*-Virulenz nicht festgestellt wurde. Interessanterweise beeinflusste der MatA Regulator, aber auch die Mat Fimbrien-Strukturgene, die Flagellenexpression: die Abwesenheit von *matA* bzw. von *matBCDEF* führte in beiden *E. coli* Stämmen zu einer Induktion der Flagellenexpression und Motilität. Der zugrundeliegende Mechanismus ist noch unbekannt.

Zusammenfassend zeigen die Ergebnisse dieser Arbeit, dass es eine beträchtliche Überlappung des Genomgehaltes und der Phänotypen bei ExPEC-Stämmen, die von Menschen oder Tieren isoliert wurden, gibt. Das Vorhandensein eines gemeinsamen Virulenzgenpools, ihre Phylogenie und ähnliche Genexpressionsprofile legen nahe, dass ein Zoonoserisiko von APEC-Isolaten ausgehen kann. Die Identifizierung bislang unbekannter Virulenzfaktoren humaner ExPEC-Stämme kann sich daher auch auf das Verständnis der Pathogenese von APEC-Isolaten auswirken. Die Ergebnisse dieser Arbeit belegen auch, wie am Beispiel der Mat Fimbrien gezeigt, dass unterschiedliche *E. coli*-Phänotypen nicht nur auf einen unterschiedlichen Genomgehalt, sondern auch auf die unterschiedliche Regulation konservierter Determinanten zurückgeführt werden kann.

2. Introduction

2.1 *Escherichia coli*, a versatile bacterium

Among bacterial infections of man and animals, those caused by *Escherichia coli* (*E. coli*) are special because of the diversity of *E. coli* pathotypes and different types of infection caused. *E. coli*, a Gram-negative bacterium of the family *Enterobacteriaceae* is a commensal bacterium of the intestinal flora in man and warm-blooded animals. It represents at least 80% of the aerobic flora. *E. coli* is a heterogeneous bacterial species with high genomic plasticity and many pathogenic variants (125, 126, 293). The different pathovars of *E. coli* are characterized by their host tropism or tissue. Some strains are responsible for intestinal infections causing severe diarrhea. Other strains cause extraintestinal infections such as urinary tract infections, newborn meningitis, septicaemia, pneumonia but also systemic infections in poultry. These bacteria must be able of infecting the host, to resist the immune system and later to efficiently colonize these different niches (164, 285) .

Among extraintestinal pathogenic *Escherichia coli* (ExPEC) strains, avian pathogenic *E. coli* (APEC) which cause several diseases in poultry, can be distinguished from uropathogenic *E. coli* (UPEC), which are responsible for approximately 90% of uncomplicated urinary tract infections (UTI) (169) and about 50 % of the nosocomial UTIs (286, 319).

APEC infection causes avian colibacillosis, a complex syndrome characterized by air sacculitis, pericarditis, peritonitis, salpingitis, polyserositis, septicemia, synovitis, osteomyelitis, and yolk sac infection (fibrinous lesions of internal organs) (118, 119). In poultry, these diseases cause important economic losses. Nowadays no effective vaccine is available on the market, antibiotic therapy remains the most appropriate treatment despite the increasing resistance to antibiotics (233, 360). Several factors involved in the pathogenesis of these bacteria have been identified and are also present in ExPEC strains, such as type I- or P- and S-type fimbriae, the siderophore aerobactin, K1 capsule or the

protein IbeA (98, 99, 153). Nevertheless, these virulence factors are not sufficient to explain the whole process of infection in poultry since some pathogenic strains have no factors above. This suggests the existence of other still unidentified virulence factors.

UTI is the most common bacterial infection in the industrialized world: in the USA 7 million patient visits per year are counted with total costs exceeding one billion dollars (17). As many as 50 % of the women report to have had at least one UTI in their lifetime (21). UTI affects either the bladder (cystitis) or the kidneys and their collecting systems (pyelonephritis), or both. The bacterial colonization of the urinary tract may be completely free of clinical symptoms ("asymptomatic bacteriuria", ABU) (364). Moreover, a pyelonephritis can be acute or chronic. The last case is a more complex disorder where the bacterial infection plays a dominant role. However, other factors like vesicourethral reflux and obstruction or immunodeficiency are also critically involved in pathogenesis. UTI is normally an ascending infection (less frequently UTI can be an infection through the bloodstream) where the bacteria derive from the patient's own faecal flora. The initial step of the pathogenesis is colonization of the distal urethra and vagina in women (305). From the urethra, the pathogens may gain entrance into the bladder. Here, when the natural defense mechanisms e.g., flushing of urine, secretion of IgA or uromucoid (specific protein, a urinary mucoprotein, insoluble, or glycoprotein of Tamm-Horsfall; type 1 pili link on it, so *E. coli* aggregate and are eliminated by urinary flux), are overwhelmed by the virulent bacteria, bacterial adhesion and colonization may occur evolving into UTI. The colonization of the urinary tract provokes cellular responses e. g., activation of the epithelial cells, secretion of cytokines and neutrophile migration into the urothelium. Therefore, the ability of some pathogens to overcome these mechanisms and colonize the urinary tract is linked to the presence of virulence factors encoded by horizontally acquired genes not present in their non-pathogenic relatives. These factors include *adhesins*, *cytotoxins*, *iron-uptake systems* and extracellular polysaccharides such as *lipopolysaccharide* and *capsules*. Nevertheless, these virulence factors, identicals to the ones found in APEC are not sufficient to explain the whole process of infection.

2.1.1 Origin of *Escherichia coli*

On July 14th, 1885, the German pediatrician Theodor Escherich described a bacterial species isolated from feces of a healthy newborn as a Gram-negative rod of about 1.1-1.5µm x 2.0-6.0 µm. Theodor Escherich considered it as a typical "colonic bacterium" and designated it "*Bacterium coli commune*". In 1919 "*Bacterium coli commune*" was re-named into *Escherichia coli* (*E. coli*) in honor of the man who discovered it. This denomination became official in 1958 on recommendation of the subcommittee *Enterobacteriaceae* of the nomenclature committee of the International Association of Microbiology Societies (28, 97).

Escherich was initially convinced that "*Bacterium coli commune*" is a "harmless commensal". However, Escherich reported "on cystitis in children provoked by "*Bacterium coli commune*" as early as 1894 in a published lecture. He hypothesised that the intestinal bacteria could be considered as a source of urinary tract infections (bladder and kidney infections). This early hypothesis that *E. coli* bacteria which persist without symptoms in the intestine and for various reasons find their way into the urinary tract where they might cause inflammation, has now been confirmed by modern biochemical and molecular biological methods (127). Lesage already suggested in 1887 that there are also pathogenic *E. coli* variants (194).

However, it was Jensen who attested *E. coli*, considered for a long time only as a commensal of the human intestine, a pathogenic role after observation of diarrhea resulting of calves infection (154). Escherich already implicitly put the potential pathogenicity of *E. coli* in observing the strong frequence of neonatal diarrhea and lethality in rabbits and guinea pigs infected by these bacteria. Escherich himself observed the morphological variety of *E. coli* colonies. Even more numerous are the serological variants. The *E. coli* strains are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profiles (168), where the specific combination of these factors defines the serotype of an isolate. *E. coli* strains of a specific serotype can be associated with certain clinical manifestations. However, the surface antigens alone are not considered to confer pathogenicity themselves. Rather there are specific clonal

lineages which have served as “hosts” for horizontally transfered virulence genes resulting in pathogenic clones (369).

According to Hacker and Kruis (126), around 50,000 different serotypes may occur in nature arising from the combination of different antigen structures. To date 173 surface (O), 80 capsular (K) and 56 flagellar (H) antigens are known in *E. coli*. In addition, there are also more than 100 adhesin variants which cause further differences in serological behavior and exhibit differences in receptor recognition.

2.1.2 Non-pathogenic

Many bacteria are normally present on the skin and mucous membranes of healthy humans and animals. They form the resident commensal flora, constituted of non-pathogenic bacteria and opportunistic pathogens. In humans, only the genital and digestive commensal flora include *E. coli*. The digestive flora is the most abundant with 500 to 1000 different bacterial species and it is the natural habitat of *E. coli* (365). At the time of birth, the intestine, which is normally sterile, is quickly colonised by micro-organisms from the environment and the urogenital tract of the mother. The bacteria start to appear in feces during the first hours after birth and their number increases gradually during the first week. The first microorganisms isolated from feces of infants are facultative anaerobic bacteria such as *E. coli* and generally belong to the *Enterobacteriaceae*, staphylococci and streptococci. In adults, *E. coli* represents about 0.1 % of the total intestinal flora and 80 % of the aerobic intestinal flora, because of 10^{11} bacteria per gram of feces with a localization mainly in terminal parts of the intestine (139, 147, 293). As in humans, the most important bacterial flora of warm-blooded animals, including poultry, is in the gastrointestinal tract, particularly in the large intestine. Together with enterococci, *E. coli* is the most widespread bacterial species among pets and are present in different proportions depending of the animal (309) (see Table 1).

Table 1: ***E. coli*** **from normal feces flora of different species (309).**

	rabbit	horse	calf	Sheep	pork	chicken	mouse	dog	cat
E. coli	2.7	4.1	4.3	6.5	6.5	6.6	6.8	7.5	7.6

Average values from 10 individuals (log CFU/g of feces)

E. coli can also be found in healthy subjects outside of its natural habitat, particularly in the upper respiratory tract of rabbits (5), of puppies (4), and chickens (76). In the avian, *E. coli* is also present on the skin and feathers (48, 49, 52, 53, 237).

The non-pathogenic strain of *E. coli* the most studied and best known is the strain K-12 and its derivatives. It was isolated in 1922 from the stool of a patient suffering from diphtheria (191) and was stored in the bacteriological strain collection of the Medical School of Stanford University in California under the laboratory designation “K-12”. When parasexual processes - i.e. the capability of exchanging genetic material between two bacterial cells - were then discovered in this *E. coli* K-12 strain, this strain became the standard research object of microbial geneticists and molecular biologists. One of the first bacterial strains whose genome was completely sequenced was consequently an *E. coli* K-12 strain (MG1655) (33).

2.2 *E. coli* Infections

Pathogenic *E. coli* bacteria are classified into different “pathotypes” according to the disease type they cause. *E. coli* strains causing intestinal infections are distinguished from other strains that are responsible for extraintestinal infections. Pathogenic *E. coli* variants are characterized by the presence of various virulence factors, such as various toxins, particular fimbrial adhesins, invasins or secretion system (see Figure 1). They can be present in the bacterial genome, encoded on genomic/pathogenicity islands, plasmids or phages.

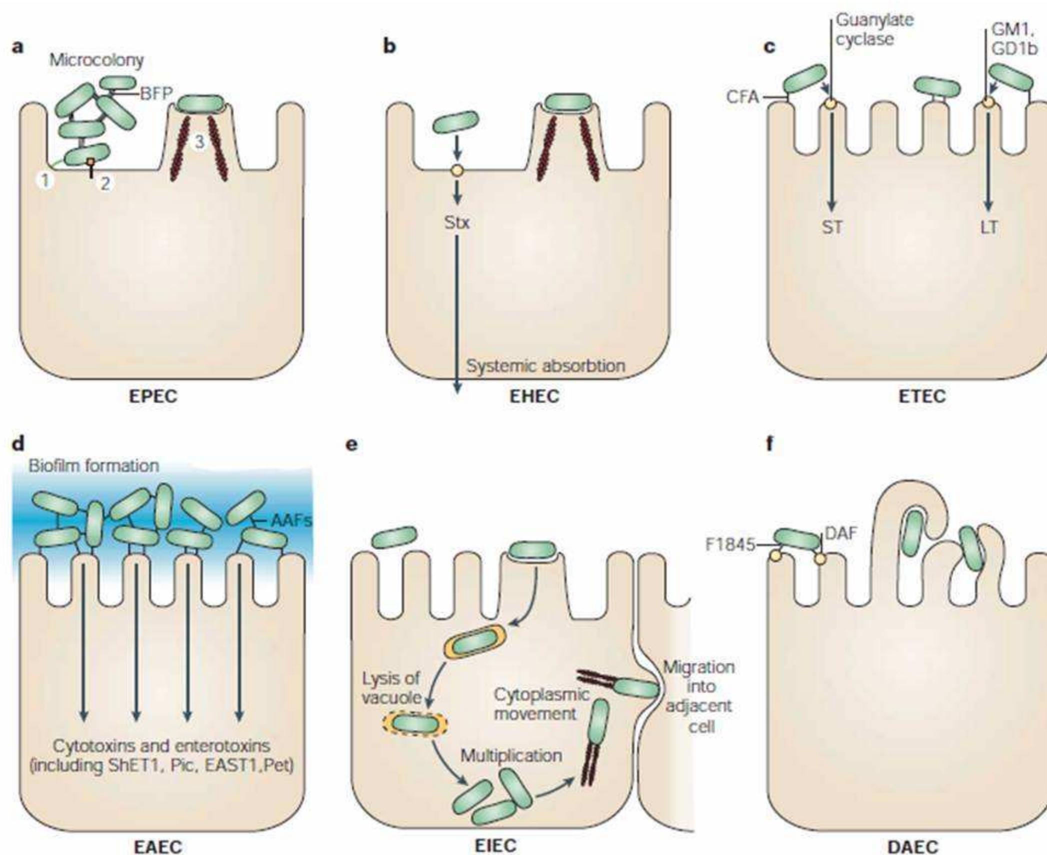


Figure 1: Pathogenicity of diarrhoeagenic *E. coli*. The six recognized categories of diarrhoeagenic *E. coli* have unique features in their interaction with eukaryotic cells. Here, the interaction of each category with a typical target cell is schematically represented. These descriptions are largely the result of *in vitro* studies and might not completely reflect the phenomena that occur in infected humans. **a** | EPEC adhere to small bowel enterocytes, but destroy the normal microvillar architecture, inducing the characteristic attaching and effacing lesion. Cytoskeletal rerrangements are accompanied by an inflammatory response and diarrhoea. 1. Initial adhesion, 2. Protein translocation by type III secretion, 3. Pedestal formation. **b** | EHEC also induce the attaching and effacing lesion, but in the colon. The distinguishing feature of EHEC is the expression of Shiga toxin (Stx), systemic absorption of which leads to potentially life-threatening complications. **c** | Similarly, ETEC adhere to small bowel enterocytes and induce watery diarrhoea by the secretion of heat-labile (LT) and/or heat-stable (ST) enterotoxins. **d** | EAEC adhere to the small and large bowel epithelia in a thick biofilm and secrete enterotoxins and cytotoxins. **e** | EIEC invade the colonic epithelial cell, lyse the phagosome and move through the cell by nucleating actin microfilaments. The bacteria might move laterally through the epithelium by direct cell-to-cell spread or might exit and re-enter the baso-lateral plasma membrane. **f** | DAEC elicits a characteristic signal transduction effect in small bowel enterocytes that manifests as the growth of long finger-like cellular projections, which wrap around the bacteria. AAF, aggregative adherence fimbriae; BFP, bundle-forming pilus; CFA, colonization factor antigen; DAF, decay-accelerating factor; EAST1, enteroaggregative *E. coli* ST1; LT, heat-labile enterotoxin; ShET1, *Shigella* enterotoxin 1; ST, heat-stable enterotoxin. (From: Kaper *et al*., 2004)

2.2.1 *E. coli* involved in intestinal infections

Intestinal pathogenic *E. coli* are subdivided at present into six pathotypes which cause diarrhoea with different clinical manifestation (164). The clinical symptoms and the virulence factors expressed by the strains, the adhesion factors and toxins, are used as criteria for their classification (see Table 2). The serotyping mainly employed in earlier years to identify and classify clinical isolates is being more and more replaced today by the molecular genetic detection of bacterial virulence and pathogenicity factor-encoding genes that are known, supported by evidence of specific pathogenic features (see Figure 1).

2.2.1.1 Enteropathogenic *E. coli* (EPEC)

EPEC was the first pathotype of *E. coli* to be described. Large outbreaks of infant diarrhoea in the United Kingdom led Bray, in 1945, to describe a group of serologically distinct *E. coli* strains that were isolated from children with diarrhoea but not from healthy children. EPEC remains an important cause of potentially fatal infant diarrhoea in developing countries (228). A characteristic intestinal histopathology is associated with EPEC infections; known as ‘attaching and effacing’ (A/E), the bacteria intimately attach to intestinal epithelial cells and cause striking cytoskeletal changes, including the accumulation of polymerized actin directly beneath the adherent bacteria. The microvilli of the intestine are effaced and pedestal-like structures on which the bacteria perch frequently rise up from the epithelial cell. The ability to induce this A/E histopathology is encoded by genes on the ‘locus of enterocyte effacement’ (LEE) (212), a 35-kb pathogenicity island (PAI). Homologues of LEE are also found in other human and animal pathogens.

Table 2: Main characteristics of the different pathovars of intestinal pathogenic *Escherichia coli*.
C: chromosomally-encoded; P: plasmid-encode

Pathotype	Adhesins	Toxins	Invasive	Histopathology	Genetic support	Host	Symptoms
ETEC	K88(F4)^{P}K99 (F5)^{P} CS31A^{P} 987P (F6)^{P} F17^{C} F18 F42 CFA/I (F2)^{P} CFA/II (F3) [CS1^{P}, CS2^{C}, CS3^{P}] CFA/III^{P} + CS6^{P} CFA/IV [CS4^{P}, CS5^{P}, CS6^{P}] CS7^{P} CS17^{P} PCFO166^{P} PCFO159^{P} PCFO148^{P}	Enterotoxin cytotoxic: LT-I^{P}, LT-II STa^{P}, STb^{P}	No	No	Chromosomal Plasmid	Human Piglet Calf Lamb	Watery diarrhoea + dehydration + acidose Newborn diarrhoea and post-weaning Watery diarrhea + dehydration + Acidose + newborn diarrhoea
EPEC	BFP^{C} FB171-14 FB171-15 FB171-16 AF/R1^{P} AF/R2^{P} intimin^{C}	EAST1$^{P/C}$	Limited	Attaching effacing (Hep-2 or HeLa)	Chromosome (LEE) Plasmid (EAF)	Human, piglet, rabbit	Watery diarrhoea + Dehydration
EHEC	Intimin^{C} Other adhesins F18 (pork)^{P}	Stx1^{C} Stx2^{C} EAST1^{C} Enterohemolysin^{P} Ehly1 Ehly2	No	Attaching-Effacing (Hep-2 or HeLa) Inflammation	Chromosome (LEE) Plasmid	Human Pork Calf	Non-bloody diarrhoea Bloody diarrhoea HUS Oedema Post-weaning diarrhoea Mucoid enteritis Bloody diarrhoea
EAEC	AAF/I^{P} AAF/II^{P}	EAST1^{P} Cytotoxins	No	Mucus biofilm Hypersecretion	Chromosome Plasmid	Human	Persistent watery and mucoid diarrhoea in adult and children
EIEC	Ipa	Enterotoxins	Yes	+/- (depends on the inflammation severity)	Chromosome Plasmid (140 MDa)	Human	Watery diarrhoea and occasionally dysentery (mucous and blood in feces)
DAEC	F1845 AIDA-I "fine projections"		Rare	?	Chromosome Plasmid	Children	Watery diarrhoea

The model of EPEC pathogenesis is considerably more complex than simple binding to epithelial cells by a single adhesin and secretion of an enterotoxin that induces diarrhoea. The emerging model, several aspects of which are reviewed elsewhere (103, 134, 170, 228), indicates that EPEC initially adhere to epithelial cells by an adhesin, the identity of which is not yet clearly established; potential candidates include BFP, the EspA filament, flagella, LifA/Efa1 and intimin (by host-cell receptors).

Diarrhoea probably results from multiple mechanisms, including active ion secretion, increased intestinal permeability, intestinal inflammation and loss of absorptive surface area resulting from microvillus effacement. EPEC are also pathogenic to animals and causes major losses in the rearing of young animals, e.g. in rabbits, chickens. EPEC have also been isolated from animals livestock with lesions A/E such as cattle (56, 110, 202, 203), pigs (368), or rabbits (217) but also among pets such as dogs and cats (42)

2.2.1.2 Enterotoxigenic *E. coli* (ETEC)

ETEC cause watery diarrhoea, which can range from mild, self-limiting disease to severe purging disease. The organism is an important cause of childhood diarrhoea in the developing world and is the main cause of diarrhoea in travellers to developing countries (228). ETEC colonizes the surface of the small bowel mucosa and secrete enterotoxins, which induce intestinal secretion. ETEC enterotoxins belong to one of two groups: the heat-labile enterotoxins (LTs) and the heat-stable enterotoxins (STs). ETEC strains might express only an LT, only an ST, or both LTs and STs. LTs are a class of enterotoxins that are closely related in structure and function to cholera enterotoxin (CT), which is expressed by *Vibrio cholerae* (311). STs are small, single-peptide toxins that include two unrelated classes — STa and STb — which differ in both structure and mechanism of action. Only toxins of the STa class have been associated with human disease (228). The STb toxin is associated with animal disease and is a 48-amino acid peptide containing two disulphide bonds (reviewed in (85)). STb can elevate cytosolic Ca^{2+} concentrations, stimulate the release of prostaglandin E2 and stimulate the release of serotonin, all of which are mechanisms that could lead to increased ion secretion.

ETEC is mainly a pathogen of developing countries, and it is well known that these countries typically have a low rate of colon cancer. Pitari *et al.* (256) have reported that STa suppresses colon cancer cell proliferation through a guanylyl cyclase C-mediated signalling cascade. Accordingly, the high prevalence of ETEC in developing countries might have a protective effect against this important disease, and indicates that infectious diseases might exist in a complex evolutionary balance with their human populations.

2.2.1.3 Enterohaemorrhagic *E. coli* (EHEC)

First recognized as a cause of human disease in 1982, EHEC causes bloody diarrhoea (haemorrhagic colitis), non-bloody diarrhea and haemolytic uremic syndrome (HUS). The bovine intestinal tract is the principal reservoir of EHEC and initial outbreaks were associated with consumption of undercooked hamburgers. Subsequently, a wide variety of food items have been associated with disease, including sausages, unpasteurized milk, lettuce, cantaloupe melon, apple juice and radish sprouts — the latter were responsible for an outbreak of 8,000 cases in Japan. Facilitated by the extremely low infectious dose required for infection (estimated to be <100 cells), EHEC has also caused numerous outbreaks associated with recreational and municipal drinking water, person-to-person transmission and petting zoo and farm visitations. A recent report indicates potential airborne transmission after exposure to a contaminated building (338). EHEC strains of the O157:H7 serotype are the most important EHEC pathogens in North America, the United Kingdom and Japan, but several other serotypes, particularly those of the O26 and O111 serogroups, can also cause disease and are more prominent than O157:H7 in many countries.

The key virulence factor for EHEC is Stx, which is also known as verocytotoxin (VT). The Stx family contains two subgroups — Stx1 and Stx2 — that share approximately 55% amino acid homology. Stx is produced in the colon and is transported by the bloodstream to the kidney, where it damages renal endothelial cells and occludes the microvasculature through a combination of direct toxicity and induction of local

cytokine and chemokine production, resulting in renal inflammation (11). This damage can lead to HUS, which is characterized by haemolytic anaemia, thrombocytopoenia and potentially fatal acute renal failure. Stx also mediates local damage in the colon, which results in bloody diarrhoea, haemorrhagic colitis, necrosis and intestinal perforation.

In addition to Stx, most EHEC strains also contain the LEE pathogenicity island that encodes a type III secretion system and effector proteins that are homologous to those that are produced by EPEC. Animal models have shown the importance of the intimin adhesin in intestinal colonization, and HUS patients develop a strong antibody response to intimin and other LEE encoded proteins. EHEC O157:H7 is believed to have evolved from LEE-containing O55 EPEC strains that acquired a bacteriophage encoding Stx (274).

2.2.1.4 Enteroaggregative *E. coli* (EAEC)

EAEC are increasingly recognized as a cause of often persistent diarrhea in children and adults in both developing and developed countries, and have been identified as the cause of several outbreaks worldwide. At present, EAEC are defined as *E. coli* that do not secrete LT or ST and that adhere to HEp-2 cells in a pattern known as autoaggregative, in which bacteria adhere to each other in a 'stacked-brick' configuration (228). Nevertheless, at least a subset of EAEC has been proven as human pathogens.

The basic strategy of EAEC infection seems to comprise colonization of the intestinal mucosa, probably predominantly that of the colon, followed by secretion of enterotoxins and cytotoxins (229). Studies on human intestinal explants indicate that EAEC induces mild, but significant, mucosal damage (140) — these effects are most severe in colonic sections. Mild inflammatory changes are observed in animal models (339) and evidence indicates that at least some EAEC strains might be capable of limited invasion of the mucosal surface (1, 24). The most dramatic histopathological finding in infected animal models is the presence of a thick layer of autoaggregating bacteria adhering loosely to the mucosal surface. EAEC prototype strains adhere to HEp-2 cells

and intestinal mucosa by virtue of fimbrial structures known as aggregative adherence fimbriae (AAFs) (64, 227, 230), which are related to the Dr family of adhesins. Several toxins have been described for EAEC. Two such toxins are encoded by the same chromosomal locus on opposite strands. The larger gene encodes an autotransporter protease with mucinase activity called Pic; the opposite strand encodes the oligomeric enterotoxin that is known as *Shigella* enterotoxin 1 (ShET1), owing to its presence in most strains of *Shigella flexneri* 2a (137, 236). Although no single virulence factor has been irrefutably associated with EAEC virulence, epidemiological studies implicate a 'package' of plasmid-borne and chromosomal virulence factors, similar to the virulence factors of other enteric pathogens. Several EAEC virulence factors are regulated by a single transcriptional activator called AggR, which is a member of the AraC family of transcriptional activators (230). One consistent observation from studies involving EAEC epidemiology is the association of the AggR regulon with diarrhoeal disease. Jiang *et al*. have recently shown that the presence of genes associated with the AggR regulon is predictive of significantly increased concentrations of faecal IL-8 and IL-1 in patients with diarrhoea caused by EAEC (155). It has been suggested that the term 'typical EAEC' should be reserved for strains carrying AggR and at least a subset of AggR-regulated genes (for which the traditional EAEC probe is an adequate marker), and that the term 'atypical EAEC' should be used for strains lacking the AggR regulon.

2.2.1.5 Enteroinvasive *E. coli* (EIEC)

EIEC are biochemically, genetically and pathogenically closely related to *Shigella* spp. Numerous studies have shown that *Shigella* spp. and *E. coli* are taxonomically indistinguishable at the species level (269, 351), but, owing to the clinical significance of *Shigella*, a nomenclature distinction is still maintained. The four *Shigella* species that are responsible for human disease, *S. dysenteriae*, *S. flexneri*, *Shigella sonnei* and *Shigella boydii*, cause varying degrees of dysentery, which is characterized by fever, abdominal cramps and diarrhoea containing blood and mucus. EIEC might cause an invasive inflammatory colitis, and occasionally dysentery, but in most cases EIEC elicits watery diarrhoea that is indistinguishable from that due to infection by other *E. coli* pathogens

(228). EIEC are distinguished from *Shigella* spp. by a few minor biochemical tests, but these pathotypes share essential virulence factors. EIEC infection is thought to represent an inflammatory colitis, although many patients seem to manifest secretory, small bowel syndrome. The early phase of EIEC/*Shigella* pathogenesis comprises epithelial cell penetration, followed by lysis of the endocytic vacuole, intracellular multiplication, directional movement through the cytoplasm and extension into adjacent epithelial cells (292). Movement within the cytoplasm is mediated by nucleation of cellular actin into a 'tail' that extends from one pole of the bacterium. In addition to invasion into and dissemination within epithelial cells, *Shigella* (and presumably EIEC) also induces apoptosis in infected macrophages (371).

Genes that contribute to this complex pathogenicity are present on a large virulence plasmid that is found in EIEC and all *Shigella* species. The sequence of the 213-kb virulence plasmid of *S. flexneri* (pWR100) indicates that this plasmid is a mosaic that includes genetic elements that were initially carried by four plasmids (46). One-third of the plasmid is composed of insertion sequence (IS) elements, which are undoubtedly important in the evolution of the virulence plasmid. This plasmid encodes a type III secretion system and a 120-kDa outer-membrane protein called IcsA, which nucleates actin by the binding of N-WASP (87, 111). The growth of actin micofilaments at only one bacterial pole induces movement of the organism through the epithelial cell cytoplasm. This movement culminates in the formation of cellular protrusions that are engulfed by neighbouring cells, after which the process is repeated. Although EIEC are invasive, dissemination of the organism past the submucosa is rare.

Although the extensively characterized type III secretion system is essential for the invasiveness characteristic of EIEC and *Shigella* species, additional virulence factors have been described, including the plasmid-encoded serine protease SepA, the chromosomally encoded aerobactin iron-acquisition system and other secreted proteases that are encoded by genes present on pathogenicity islands.

2.2.1.6 Diffusely adherent *E. coli* (DAEC)

DAEC are defined by the presence of a characteristic, diffuse pattern of adherence to HEp-2 cell monolayers. DAEC have been implicated as a cause of diarrhoea in several studies, particularly in children >12 months of age (228, 294). Approximately 75% of DAEC strains produce a fimbrial adhesin called F1845 or a related adhesin (Ref. 29; unpublished observations in review 164); F1845 belongs to the Dr family of adhesins, which use DAF, a cell surface glycosylphosphatidylinositol-anchored protein, which normally protects cells from damage by the complement system, as the receptor (27, 133, 252). DAEC strains induce a cytopathic effect that is characterized by the development of long cellular extensions, which wrap around the adherent bacteria (see Figure 1). This characteristic effect requires binding and clustering of the DAF receptor by Dr fimbriae (27). All members of the Dr family (including UPEC as well as the DAEC strain C1845) elicit this effect (29). Binding of Dr adhesins is accompanied by the activation of signal transduction cascades, including activation of PI-3 kinase (252). Peiffer *et al.* have reported that infection of an intestinal cell line by strains of DAEC impairs the activities and reduces the abundance of brush border-associated sucrase isomaltase and dipeptidylpeptidase IV (251). This effect is independent of the DAF-associated pathway described above, and therefore provides a feasible mechanism for DAEC-induced enteric disease and also indicates the presence of virulence factors in DAEC other than Dr adhesins. Tieng *et al.* (323) have proposed that DAEC might induce expression of MICA by intestinal epithelial cells, indicating that DAEC infection could be proinflammatory; this effect could potentially be important in the induction of inflammatory bowel diseases.

2.2.2 Extraintestinal pathogenic *Escherichia coli*

Extraintestinal infections involving *E. coli* include urinary tract infections, newborn meningitis as well as human and animal septicemia. Unlike IPEC, ExPEC are very often found as intestinal commensal flora and are not the cause of gastroenteritis in humans. The acquisition of ExPEC by the host does not often cause an infection, they

will have to colonize first, from the intestine or external middle, tissues and organs normally sterils (urinary tract, peritoneal cavity, lungs) (158, 288)

ExPEC strains express different types of virulence factors which allow them to colonize the surface of the mucous membranes of the host, to escape host defence mechanisms, to multiply under conditions of limited essential elements (nutrients) such as iron. Other virulence factors will enable ExPEC to invade the host tissue and to induce an inflammatory response (156). These virulence factors of ExPEC include various adhesins, surface polysaccharides (capsule, LPS), toxins, siderophores, proteases, invasins and proteins enabling them to resist the effects of complement. It is impossible today to characterize with precision different pathotypes among ExPEC solely on the basis of their virulence factors. Nevertheless, ExPEC strains were isolated from various extraintestinal infections. There are thus *E. coli* responsible for urinary tract infections (UPEC, Uropathogenic *E. coli*), *E. coli* responsible for the newborn meningitis (NMEC, Neonatal Meningitidis *E. coli*) and *E. coli* implicated in localized and systemic poultry infections (APEC Avian pathogenic *E. coli*).

2.2.2.1 The UPEC pathotype

The urinary tract is the most common site of bacterial infection in industrialized countries (348), and urinary tract infection (UTI) is also the leading nosocomial disease. UTI can be caused by several microbial pathogens. The most common causative agent of urinary tract infections, however, are uropathogenic *E. coli* (UPEC), which cause uncomplicated UTI in about 80% of all cases (148, 319).

The urinary tract represents a usually sterile compartment, which is protected from bacterial infections by various mechanisms such as urine flow and immune responses. Furthermore, the urinary tract is a hostile environment in terms of supporting bacterial growth. The chemical composition, osmolarity, and pH of urine determine the rate of bacterial growth and the maximum population that can be supported, and can be very variable, depending on the diet. Normal urine constituents include amino acids and

glucose, which are usually present at sufficient concentrations to support rapid bacterial growth. However, other components of urine, such as urea and organic acids, may inhibit

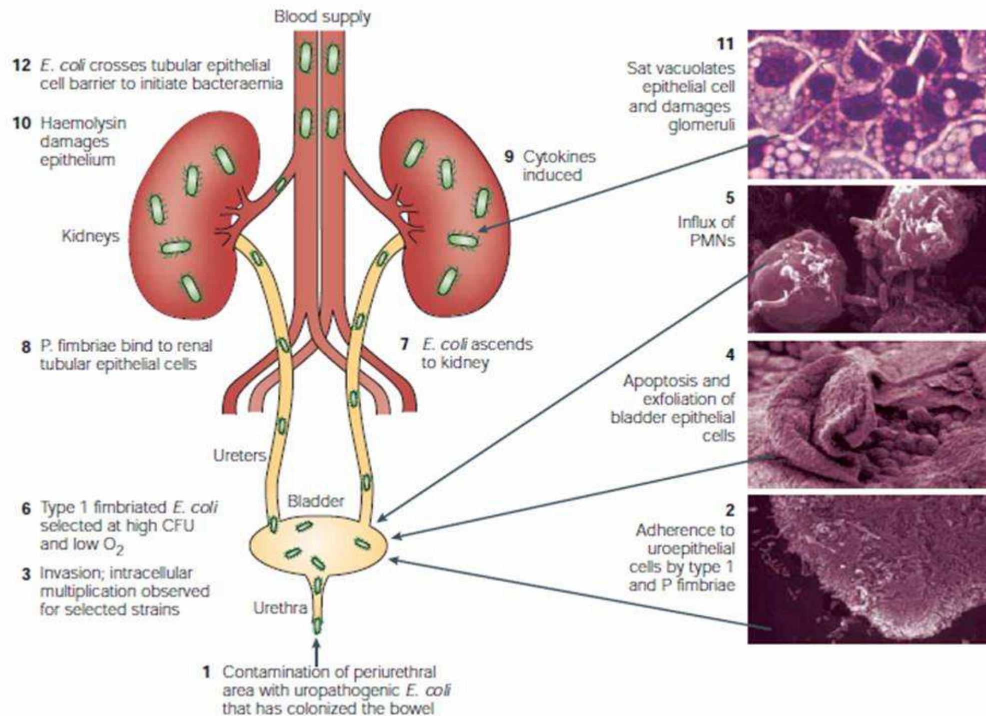


Figure 2: Pathogenesis of urinary tract infection caused by uropathogenic *E. coli*. The figure shows the different stages of a urinary tract infection. CFU, colony-forming units; PMNs, polymorphonuclear leukocytes. (From: Kaper *et al*., 2004)

growth, mainly by affecting pH and osmolarity (14). Therefore, the ability of some pathogens to overcome these mechanisms and colonize the urinary tract is linked to the presence of virulence factors encoded by horizontally acquired genes not present in their non-pathogenic relatives. These factors include *adhesins*, *cytotoxins*, *iron-uptake systems* and extracellular polysaccharides such as *lipopolysaccharide* and *capsules*. For comprehensive reviews on virulence factors of uropathogenic *E. coli* see Emödy *et al.* (2003), Johnson (1991), Mühldorfer *et al*. (2001), or Oelschlaeger *et al*. (2002).

The subset of *E. coli* that causes uncomplicated cystitis and acute pyelonephritis is distinct from the commensal *E. coli* strains that comprise most of the *E. coli* colonizing the lower colon of humans. *E. coli* from a small number of O serogroups (six O groups cause 75% of UTIs) have phenotypes that are epidemiologically associated with cystitis

and acute pyelonephritis in the normal urinary tract, which include expression of P fimbriae, α-haemolysin, aerobactin, serum resistance and encapsulation. Clonal groups and epidemic strains that are associated with UTIs have been identified (238, 255). Availability of the genome sequence of *E. coli* CFT073 (353) and 536 (45), efforts by other investigators to identify virulence genes by signature-tagged mutagenesis (19) and other methods have allowed the development of a model of pathogenesis for UPEC (see Figure 2).

2.2.2.2 Meningitis/Sepsis-associated *E. coli* (MNEC, SEPEC)

E. coli is the second leading cause of meningitis among newborns (25-30% of cases) after the group B streptococci (210), with a case fatality rate of 15–40% with severe neurological defects in many of the survivors (71, 333).

Sepsis-causing *E. coli* employ pathogenicity factors similar to UPEC. Sepsis caused by *E. coli* occurs in humans, cattle, sheep, pigs and poultry. Septicemic *E. coli* (SEPEC) protect themselves from attack by the complement system by having certain capsule types and, to a lesser extent, long-chain lipopolysaccharides. This enables them to survive for a longer time in blood serum. SEPEC are therefore described as "serum-resistant". In humans they are transferred from the mother to the neonate during birth and can trigger meningitis in the newborn child or by exposure to bacteria during a hospital stay (104, 239). The *E. coli* meningitis usually develops through various stages of interaction between the host and the bacterium. These mechanisms include MENEC colonization of the respiratory or digestive tract, the invasion of intravascular space followed by a survival and an increase in the blood. This strong bacteremia is necessary for *E. coli* to cross the blood-brain barrier, invade the central nervous system and thus cause meningitis (173, 176).

MNEC also protect themselves by capsule formation (often of the K1 serotype). They adhere to epithelial and endothelial cells using e.g. S- or F1C fimbrial adhesins and can penetrate through these tissue barriers. As with *E. coli* pathotypes that have a well

defined genetic basis for virulence, strains that cause meningitis are represented by only a limited number of O serogroups, and 80% of the strains are of the K1 capsule type. One interesting difference between MNEC and *E. coli* that cause intestinal or urinary tract infections is that although the latter strains can be readily transmitted by urine or faeces, infection of the central nervous system offers no obvious advantage for the selection and transmission of virulent MNEC strains.

As for other *E. coli* pathotypes, the genomes of these *E. coli* K1 strains have additional genes that are not found in the commensal *E. coli* K-12 strains. In genomic comparisons, the genome of *E. coli* RS218, a newborn meningitis isolate, was found to have at least 500 kb of additional genes inserted in at least 12 loci compared with *E. coli* K-12 (38, 279). In addition, strain RS218 harbours a 100-kb plasmid, on which at least one virulence factor has been localized (18). Some insights into the mechanism of pathogenesis of these strains have been obtained. K1 strains use S fimbriae to bind to the lumenal surfaces of brain microvascular endothelium in neonatal rats (248). Invasion requires the outer-membrane protein OmpA to bind to the GlcNAcβ1-4GlcNAc epitope of the brain microvascular endothelial cell receptor glycoprotein (265). Other membrane proteins — for example, IbeA, IbeB, IbeC and AslA — are also required for invasion (174). Invasion correlates with microaerobic growth and iron supplementation (117). The toxin CNF1, which has been shown to induce bacterial phagocytosis in epithelial cells (100), is required for invasion of human brain microvascular endothelial cells and may involve the same mechanism (18), as is the K1 capsule, which elicits serum resistance and has antiphagocytic properties. In an experimental model, strains that express K1 capsule proteins and those that do not were able to cross the blood-brain barrier, but only the K1-expressing strains survived (142). As a consequence of invasion, actin cytoskeletal rearrangement occurs and tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin is induced (272). In addition, a substantial list of *in vivo*-induced genes, including those that encode iron-acquisition systems, was compiled using *in vivo* expression technology (IVET) in conjunction with a murine model of septicaemic infection (172).

2.2.2.3 The APEC pathotype

In birds, *E. coli* is a normal resident of the intestinal flora, the upper respiratory tract (trachea, pharynx) and is also found on the skin and feathers (20, 76). However, some *E. coli* strains, so-called avian-pathogenic *E. coli* (APEC), can cause infection in poultry. APEC strains comprise a subset of pathogenic *E. coli* that cause extraintestinal diseases in poultry (118). The most common syndrome starts as an infection of the upper respiratory tract in 3 to 12-week-old broiler chickens and turkeys, and frequently develops into a systemic infection. APEC infections are enhanced or iniated by predisposing factors such as environmental conditions and viral or *Mycoplasma* infections (77, 119).

In poultry, APEC have been associated with various extraintestinal infections. The most common are the respiratory colibacillosis, necrotizing dermatitis syndrome and the Swollen Head Syndrome (20, 77, 119). The respiratory colibacillosis is one of the leading causes of mortality and disease in poultry, and causes economic losses in the poultry (20).

The serological tests of somatic antigens are the classical characterization method of APEC strains. Early studies conducted in 1961 by Sojka and Carnaghan from 243 strains of *E. coli* isolated from internal organs of birds suffering of colibacillosis showed that among APEC the most frequently serogroups encountered are O1 (Ol: Kl), O2 (O2: Kl) and O78 (O78: K80)(306). In the various studies that have followed, these three serogroups always represent the majority serogroups of APEC (32, 77, 281). However, an increasing variety of serogroups identified and the large number of non-typable strains make it difficult to carry out a relevant APEC classification based on serotyping.

A) Localized avian colibacillosis

Omphalitis (umbilic infection) may be technically defined as an inflammation of the navel. As commonly used, the term refers to improper closure of the navel with subsequent bacterial infection (navel ill; mushy chick disease). This also refers to the

yolk sac infection. The contamination happens by the feces, during the entry through the unhealed navel or penetration of the egg shell prior to or during incubation. This results in a high mortality of the young birds until 3 weeks after birth (20).

The necrotizing dermatitis is a skin inflammation. Although rare in animals, they are very frequent in birds. The injuries may reach the muscle, and are often associated with abscess formation (190). They are often localized in the cloacal and abdominal zone of the birds (89-92).

The swollen head syndrome (SHS) maybe observed in chicken and turkey. SHS is usually a multifactorial disease; it is believed that the initial lesions are caused by viruses.

The ovaritis and salpingitis (oviduct inflammation) are found in the adult chicken. They are due to an ascending infection from the cloaca and the left abdominal air sac. The layer which had these injuries may contaminate the eggs before the shell formation, and be responsible for high embryo mortality. A peritonit may be due to a salpingitis, or not (20).

B) Systemic colibacillosis with respiratory origin

The systemic colibacillosis with respiratory origin is the most frequent form of colibacillosis in the chicken. It may develop into bacteremia and septicemia with a relatively high mortality rate. The respiratory colibacillosis is essentially present in the animals between 3 and 12 weeks, with important losses between 4 and 9 weeks (77). Colibacillosis is typically characterized by an air saculitis: the air sacs (see Figure 3) are thicker than normal, and appear white or opaque rather than transparent with accumulation of material (fibrine). In the generalized form, fibrinous injuries of the serous membrane can be observed.

The respiratory tract, which is the principal entrance way for the bacteria, plays a crucial role in the pathogenesis. The healthy animal has few phagocytes present in the respiratory tract like neutrophil and heterophil granulocytes or macrophages (329). The

pathogens enter by inhalation of contaminated dust particles with *E. coli* originating from the intestinal tract of healthy or ill animals. Other biological agents may enhance APEC infection. The infectious bronchitis virus (IBV) causes an economically important respiratory disease in poultry worldwide, as well as the Newcastle disease virus or the causative agent of Gumboro disease, *Mycoplasma gallisepticum*; but also non-biological factors like the ammonium concentration or the dust in the rearing (20). Their inhalation decreases the number of the mucus secretory cells and the deciliation of epithelial cells. This decrease of mucus allows the APEC to adhere to the epithelium and persist in the respiratory tract of the animal (226).

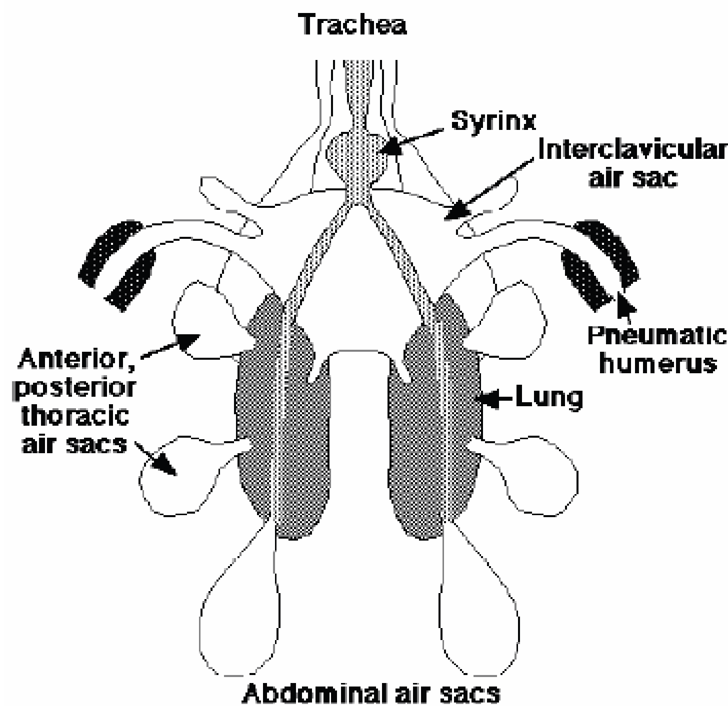


Figure 3: Scheme of the avian respiratory system. Chickens have a total of nine air sacs: four connected to each lung (two cervical sacs, two anterior thoracic sacs, two posterior thoracic sacs, two abdominal sacs), and one large intraclavicular air sac shared between the two lungs. (From: http://www.hiyt.afhe.ualberta.ca/winter06projects/breathbones.pdf)

In the lower part of the respiratory tract, lungs and air sac, few APEC strain may resist inside the macrophages (258). Mellata *et al.* demonstrated the role of type I fimbriae in the association of APEC with phagocytes, whereas the K1capsule, the O78 LPS antigen and P fimbriae allowed the bacteria to escape phagocytosis (216). Like it,

the APEC may colonize the respiratory tract and propagate in the rest of the organism. This form of colibacillosis may be reproduced experimentally either by intra-tracheal inoculation of the pathogen or by inoculation directly into the airsac (75, 258).

2.3 Virulence factors of ExPEC

Different potential virulence factors were identified in ExPEC, allowing them to adhere, to penetrate the epithelial cells, to resist to the immune system and to multiply. In Table 3, you may see a list of the differents virulent factors clearly identify in ExPEC.

2.3.1 Fimbrial adhesins

The fimbriae possess fibre-like structures and are visible on the bacteria surface by electronic microscopy. Fimbriae exhibit a composite structure, consisting of a rod-shaped shaft of 6-7 nm in diameter comprising over a thousand major subunits and minor subunits.

In ExPEC, we can distinguish four important types of fimbriae, i.e. type 1 fimbriae, P fimbriae, S/F1C fimbriae (and AC/I fimbriae). P-, S- and F1C-fimbriae are more exclusively associated with extraintestinal *E. coli* isolates and the tip of these adhesins recognize carbohydrate moieties: Galα(1-4)Gal, α-sialyl-2,3-β-galactose, and GalNAcβ(1-4)Galβ, respectively. These fimbriae are factors contributing to the virulence potential of such strains, but they are not necessarily sufficient to cause disease (219).

2.3.1.1 Type 1-fimbriae

The type 1-fimbriae are extracellular structures encoded by a group of nine genes localized on the core chromosome (*fimA* to *fim I*) where seven genes are organized in an operon (*fimA*, *fimC*, *fimD*, *fimF-I*) whose expression is phase variable (88, 105, 211).

Table 3: Virulence factors of extraintestinal pathogenic *Escherichia coli*

Virulence factors	Role	Gene
Adhesins and invasins		
Type 1	Adhesion to the respiratory tract (APEC) or uroepithelial cells (UPEC)	*fimA-H*
Dr		*afa/dra*
P	Adhesion to depth organs (APEC) or uroepithelial cells (UPEC)	*papA-K*
S-/F1C	Adhesion to depth organs (APEC)	*sfaA-G, focA-G*
F17	or uroepithelial cells (UPEC)	*f17ACDG*
Curli	Colonization factor	*csgA-G*
IbeA	Promotes invasion	*ibeA*
Iron acquisition systems		
Aerobactin	Capture iron in the host	*iucA-D/iutA*
Salmochelin		*iroBCDN*
Yersiniabactin		*irp2 and fyuA*
IreA		
Chu		
Pst System	Phosphate ATP-dependant transporter	*phoU-pstSCAB*
Tsh protein	Proteolytic activity	*tsh*
Immune resistance system		
K1Capsule	Protection against the serum (complement inhibition),	*kpsMT-neuDBACES* *kpsFEDUCS*
Outer membrane protein A	protection against phagocytosis	*ompA*
		traT
Iss		*iss*
LPS (O78)		*rfb* locus
Toxin and cytotoxin		
α-haemolysin	Cell lysis	*hlyA*
Colibactin		pks genomic island
CNF	Altered cytoskeleton, necrosis	*cnf*
CDT	DNaseI activity, blocks mitosis in G2/M phase	*cdtABC*
Sat	Vacuolation	*sat*
Verotoxin	Cell damage	*vt2y*
Vat	Vacuolation	*vat*

The *fimA* gene encodes the major component of the structure, the fibrillin. Other genes of the operon encode minor proteins, including *fimH* encoding the adhesin (205). The role of the major subunits is yet unclear, although they have been proposed to be important for adherence to mammalian extracellular matrix proteins (180).

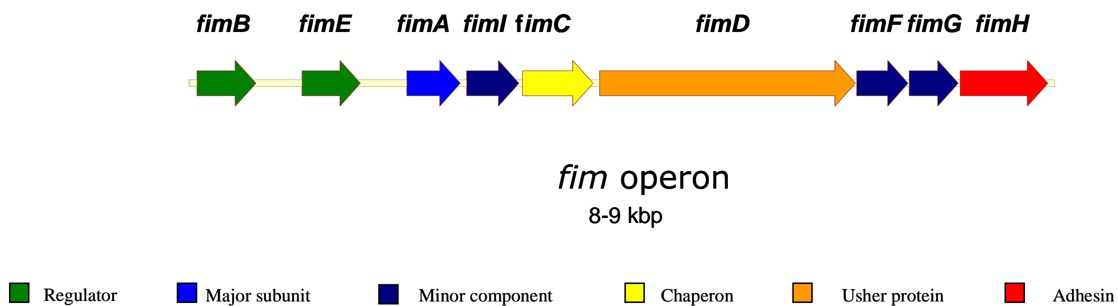


Figure 4: **Genetic organization of the *fim* locus coding for type 1-fimbriae.**

The adhesin and some other minor subunits are responsible for the specific binding to carbohydrate moieties on the surface of eukaryotic cells, therefore contributing to specific adherence. The synthesis, export, correct folding and ordered assembly during the fimbrial biogenesis occurs in a coordinated manner (302). These fimbriae are characterized by their ability to adhere to and agglutinate erythrocytes of mammals and birds. This adhesion is inhibited by the addition of D-mannose which blocks the adhesin FimH. Accordingly, these fimbriae are also called mannose-sensitive hemagglutinating (MSHA) fimbriae (84, 341). Type 1-fimbriae are present in both ExPEC and non-pathogenic strains. However, these fimbriae are more represented among pathogenic than among non-pathogenic strains (75.45% vs 55.5% (83) 100% vs 40% (362), 90.4% vs 26.7 (214) and have been for a long time considered as potential virulence factors for ExPEC.

During UTI, between 4 and 24 hours after infection, the new environment in the bladder selects for the expression of type 1-fimbriae (122), which play an important role early in the development of an UTI (62). Type 1-fimbriated *E. coli* attach to mannose moieties of the uroplakin receptors that coat transitional epithelial cells (225). Attachment triggers apoptosis and exfoliation; for at least one strain, invasion of the bladder epithelium is accompanied with formation of pod-like bulges on the bladder surface that

contain bacteria encased in a polysaccharide-rich matrix surrounded by a shell of uroplakin (10). In strains that cause cystitis, type 1-fimbriae are continuously expressed and the infection is confined to the bladder (62).

Among APEC expressing type 1-fimbriae, the *in vivo* expression of these adhesins was highlighted in the trachea, the air sacs and lungs, but not in the blood or deep organs. This result suggested a potential role in the early stages of infection (80, 259). Several experiments have shown, however, that these fimbriae are not indispensable for the colonization of the upper respiratory tract poultry by APEC and that on the contrary, the deletion of the gene coding the adhesin FimH promotes colonization of the trachea by the APEC (13, 204).

In addition it was shown that type I-fimbriae promote phagocytosis by macrophages and heterophils in chickens (216). A correlation between the presence of these fimbriae and resistance of bacteria to bactericidal serum has been suggested (83, 362). However, these observations cannot be generalized since the discovery of a type 1-fimbriae-negative APEC strain which still has the ability to resist complement. This suggests a minor role of these fimbriae for serum resistance of APEC strains (215, 259). The ro1e of type 1-fimbriae in the pathogenesis of avian colibacillosis has so far not been demonstrated. However, recent studies have shown that they could be involved in the invasion of human intestinal cells in culture (39).

2.3.1.2 P-fimbriae

Many ExPEC strains express P-fimbriae which are one of the most extensively studied adhesin, and also the first virulence-associated factor identified for UPEC. They were first described in *E. coli* isolated from urinary tract infections (pyelonephritis) in humans (162). P-fimbriae are heteropolymers encoded by a chromosomal locus of 11 genes (*papA* to *papK*) (151, 166) (Figure 5). The *pap* locus codes for the major protein PapA and the adhesin PapG that exists in three variants (317). These variants recognize different glycolipid isoreceptors and are recognizable by their ability to agglutinate

different types of erythrocytes. As this haemagglutination is not inhibited in the presence of D-mannose, these fimbriae are also designated "mannose-resistant hemagglutination (MRHA) fimbriae" (334).

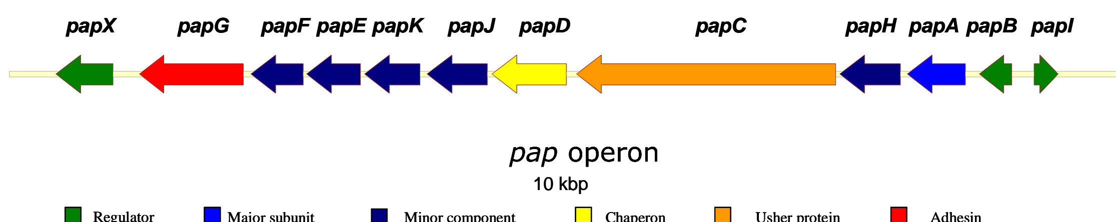


Figure 5: Organisation of the *pap* locus coding for the P-fimbriae in *E. coli* UTI89.

P-fimbriae, encoded by the *pap* (pyelonephritis-associated pili) genes, are significantly prevalent among strains of UPEC that cause pyelonephritis and are characterized by their adherence to Gal(α1–4)Galβ moieties present in the globoseries of membrane glycolipids on human erythrocytes of the P blood group and on uroepithelial cells (192, 193). The PapG variant and the chromosomal location of *pap* alleles typically differ among UPEC strains. The *pap* gene clusters reside within pathogenicity islands.

Since the discovery of P-fimbriae, it has been hypothesized that these adhesins contribute to the pathogenesis of UPEC within the mammalian urinary tract. An earlier study, conducted in 1987, demonstrated that the serum of female patients with symptoms of pyelonephritis contained P-fimbrial antibodies, suggesting that P-fimbriae were expressed during infection (72). Similarly, another study conducted shortly thereafter demonstrated that bacteria obtained from midstream or catheterized urine specimens from patients with *E. coli* cystitis expressed type 1- and P-fimbriae (177). Thus, both studies provided compelling evidence for the *in vivo* expression of P-fimbriae during human UTI. To determine whether P-fimbriae are indispensable for UPEC pathogenesis, isogenic P-fimbrial mutants of different UPEC strains have been constructed and studied in different animal models of ascending UTI. It was demonstrated that after one week of infection, no significant differences in bacterial load or histological findings between the wild-type and double-*pap* mutant were detected in the urine, bladder, or kidney at any challenge concentration. But none of these experiments could fully satisfy the molecular

Koch's postulates. More recent studies have uncovered a molecular crosstalk between the Toll-like receptor 4 that binds bacterial lipopolysaccharide and P-fimbrial-mediated attachment, which is lipopolysaccharide-independent. Activation of the Toll-like receptor 4 by P-fimbrial attachment subsequently leads to the production of pro-inflammatory cytokines and chemokines (interleukin-6 and CXCL8, respectively) and recruitment of neutrophils (25). Since P-fimbriae are implicated in triggering inflammation, it can be deduced that they may also contribute to the pathology and symptoms of acute pyelonephritis. It appears that there is a subtle role for P-fimbriae in mediating adherence to uroepithelial cells *in vivo* and establishing a robust inflammatory response during renal colonization, which in turn contributes to kidney damage during acute pyelonephritis.

The APEC strains produce mainly P-fimbriae belonging to serotype F11 fimbriae close to the same type associated with UPEC (336). As for type l-fimbriae, the expression of P-fimbriae is subject to phase variation (337, 355). P-fimbriae are more frequently present in strains isolated from septicaemic chickens than among non-pathogenic strains (69.1% vs 14.3 (83), 41.2% vs 15.6% (214)). As for type 1-fimbriae, the role of type P-fimbriae in the pathogenesis of APEC is not yet well defined. Several studies have shown that these fimbriae did not participate in the adhesion of the bacteria to the pharynx and trachea cells *in vitro* (336, 342), or the adhesion of bacteria *ex vivo* on trachea cuts of chicken (80). These observations suggest that P-fimbriae receptors are absent from the surface of these cell types or their expression in the upper respiratory tract requires special conditions. However, chickens inoculated intra-tracheally or in the air sacs with an APEC strain with fimbriae F11 show a specific anti-F11 answer characteristic of P-fimbriae expression *in vivo*. The expression of P-fimbriae has also been demonstrated *in vivo* by immunofluorescence in blood and some deep organs (kidney and heart) (259, 260). These observations suggest the role of P-fimbriae in the colonization of organs and the development of sepsis (259, 260). Although no difference has been observed upon the colonization of organs and multiplication in the blood of a wildtype APEC strain and its mutant *papG*$^-$ (215), another study which compared a *pap*$^+$ APEC strain and its *pap*$^-$ mutant described the role of P-fimbriae for bacterial attachment phagocytes thus demonstrating their involvement in the resistance to phagocytosis (216).

These results suggest that P-fimbriae provide an advantage to bacteria during infection and play a role rather in the final stages of infection, possibly in the persistence of the bacterium in the animal, and resistance to phagocytosis (260, 315).

2.3.1.3 S-/F1C-fimbriae

The fimbriae of the S-/F1C-family include several fimbrial type, e.g. S-, F1C- and AC/I-fimbriae (171). S-fimbriae are coded by a locus of nine genes (*sfaA* to *sfaH*; Figure 6). They are composed of the major subunit SfaA and the minor subunits SfaG, SfaH and adhesin SfaS. The adhesin SfaS adheres to α-sialyl-2,3-β-galactose containing glycoproteins present on the surface of eukaryotic cells, while the major subunit SfaA also has adhesive properties and can bind to the sulfated glycolipids present on the epithelial cells of the brain (246, 264).

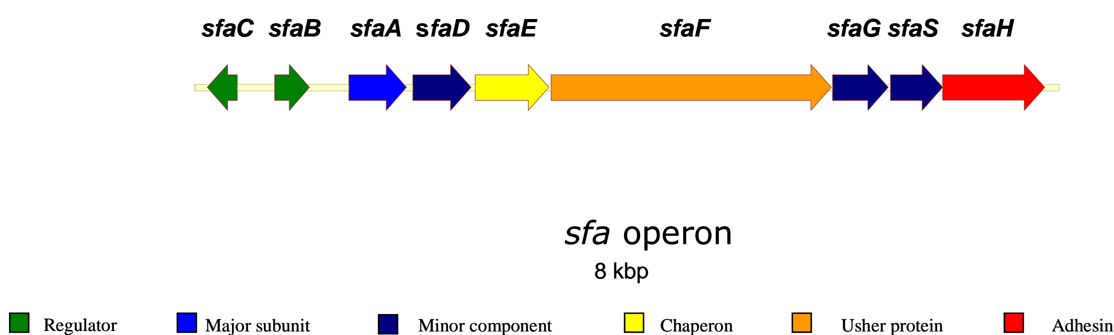


Figure 6: Organisation of the *sfa* region coding for the S-fimbriae in *E. coli* UTI89.

S-fimbriae have been known to mediate adherence of UPEC to uroepithelial cells *in vitro* and *in vivo*, suggesting their involvement in infection. Between 30-60% of the UPEC express S-fimbriae (218). S-fimbriae are associated with neonatal sepsis and meningitis (181). Studies suggest that adhesion of S-fimbriated bacteria to the binding sites observed in the neonatal brain plays a role during bacterial invasion from circulation into the cerebrospinal fluid (248).

Similar structures of the S-family fimbriae have been identified among APEC and named AC/I (avian *E. coli* I) (231, 367). The analysis of the genetic organization of the *fac* operon (fimbriae of avian pathogenic *E. coli*) coding for AC/I fimbriae confirmed that

they belonged to the family of S-fimbriae with an identical organization relative to that of the *sfa* operon (16). These fimbriae are present only in a few APEC (about 4.4% of strains studied) (73, 178, 316). AC/I fimbriae have no hemagglutinating properties but adhere to the cells of the trachea, suggesting their involvement in the infection (367).

2.3.2 Curli fimbriae

Curli are thin and filamentous structures present on the surface of *E. coli* and *Salmonella* spp. (282). These fimbriae are encoded by seven genes clustered in two operons transcribed in the opposite directions: *csgDEFG* and *csgABC* (130). The protein CsgA (curlin) is the major curli subunit and is secreted in a soluble form into the extracellular environment. CsgB, located on the surface of the bacterial membrane is involved in the polymerization of CsgA monomers by "nucleation-precipitation" (131). CsgE and CsgF participate in polymerization of CsgA monomers (57) and are necessary to assemble an effective curli fimbriae (278) (Figure 7).

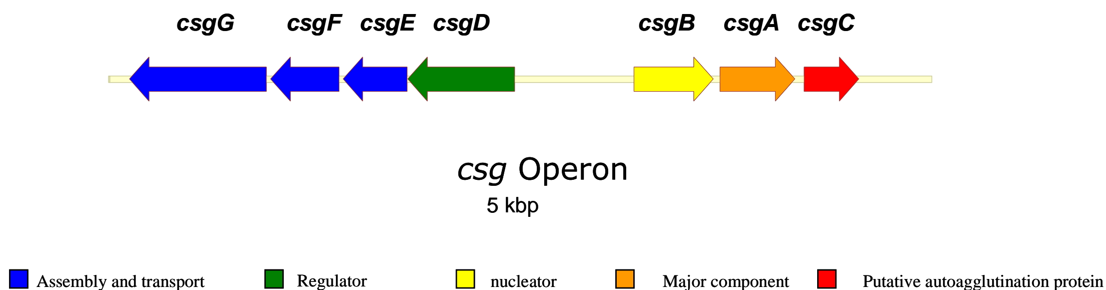


Figure 7: Organisation of the *csg* genes coding for curli in *E. coli*

Curli are present on the surface of ExPEC strains and non-pathogenic strains (209, 267). The role of curli in the adhesion and the formation of *E. coli* biofilms was highlighted first in 1998 by the characterization of an overproducer of curli (340). Further studies of adhesion properties of this overproducer mutant have proven its ability to colonize surfaces such as glass, polystyrene or sand (43). Among APEC, curli can be involved in the invasion of several types of eukaryotes cell by strains of serogroup O78 (113, 114) but their role in the avian colibacillosis is not clarified .

In UPEC, it is suggested that these fimbriae play a role only in the early phase of infection (e.g., adherence to periurethral skin surface), since they are frequently expressed only at 30 °C (242) . In the last years, isolates have been detected in which co-expression of curli fimbriae and cellulose occurs at 30 °C as well as at 37 °C (rdar morphotype), but the importance of this trait for the survival and colonization in the host organism remains unclear (370).

2.3.3 The IbeA protein

The role of the IbeA protein was first highlighted in NMEC strain RS218: an *ibeA*:Tn*phoA* mutant invaded less efficiently human brain microvascular endothelial cells (BMEC) than the wild-type (35). These early observations were confirmed later by the construction of a mutant deleted of *ibeA* gene (149).

The prevalence of gene *ibeA* was analysed among APEC strains and non-pathogenic for poultry (107). None of the non-pathogenic strains of *E. coli* had *ibeA*. A small proportion of APEC strains had *ibeA* (26%), but this gene was strongly associated with strains of serogroup O88 (100%), Ol8 (70% -100%) and O2 (49.1%) (107, 222). The IbeA protein of APEC strain BEN2908 similar to that of the strain RS2l8 was involved in the invasion of BMEC (222). Its precise role remains to be clarified although interactions between IbeA and eukaryotic proteins have been detected. In an experimental model of chicken infection the involvement of IbeA in the avian colibacillosis (bacteremia and colonization of the liver significantly lower for the mutant strain than for the wild-type) has been shown (107, 222). The authors suggest that IbeA participates in the crossing of bird epithélia, the exact location, however, remains to be determined.

2.3.4 Iron acquisition systems

Iron is necessary for growth of most microorganisms. Bacteria need about 10^{-6} mol/L for their growth. The concentration of free iron available in body fluids animals and humans is much lower (about 10^{-18} mol/L) and does not cover the needs of the

bacterium (59). To remedy this lack, bacteria have developed two strategies to dispose of iron present in eukaryotic cells. On the one hand, bacteria express receptors which can bind complexed iron as present in the host organism in proteins such as transferrin, lactoferrin but also in hemoglobin. Then, the bacteria can take this iron up and use it for growth. The second strategy is the synthesis of siderophors with high affinity for iron, allowing them to capture iron ions by competing with physiological chelators (271).

The genes coding for the biosynthesis of such iron-uptake systems in *E. coli* may be located on plasmids or on the chromosome. The gene clusters encoding the enzymes for enterobactin (*ent*) and the ferric dicitrate transport system (*fec*) have a commonly conserved localization in the *E. coli* core genome. However the *fec* gene cluster has been identified to be PAI-encoded in *Shigella flexneri* (198).

Another mechanism for iron acquisition in pathogenic *E. coli* is the direct utilization of host iron compounds, particularly heme or hemoglobin (187, 188). Hagan *et al.* demonstrate the importance of others iron acquisition factors on multiple culture conditions designed to mimic the *in vivo* environment of the pathogen. Iron compound receptors FhuA, IutA, IroN, ChuA, Iha, and IreA were detected, and a novel iron-related OMP was also identified, hypothetical protein c2482. The genes encoding ChuA, IroN, hypothetical protein c2482, and IutA are significantly more prevalent ($P < 0.01$) among UPEC strains than among fecal-commensal *E. coli* isolates (128).

E. coli produces two main types of siderophors, the catechole enterobactin and the hydroxamate aerobactin (produced especially by invasive strains of *E. coli*) (40). In addition to these two siderophore types, two other iron acquisition systems are present in several *E. coli* (salmochelins, yersiniabactin, the Sit and the Fec system).

Aerobactin seems to have an important role in the pathogenesis of avian colibacillosis (108). The aerobactin system is encoded by an operon of five genes (*iucA*, *iucB*, *iucC*, *iucD* and *iutA*) located on type ColV plasmids or on chromosome (30, 208).

The genes *iucA-D* encode enzymes necessary for the synthesis of aerobactin while *iutA* codes for an outer membrane receptor (Figure 8).

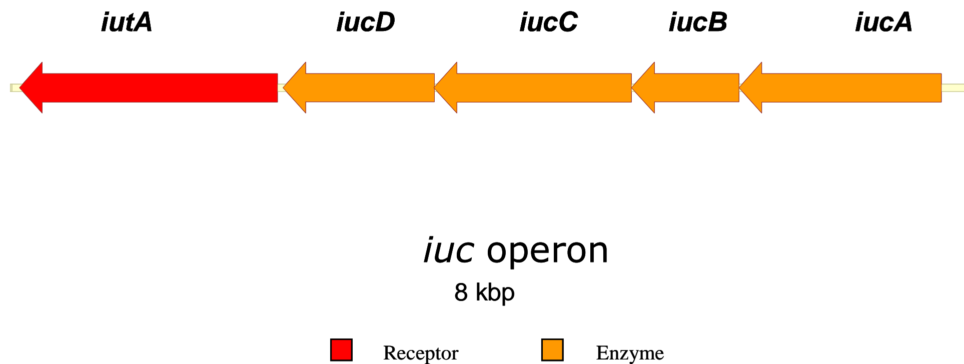


Figure 8: Genetic organisation of the genes coding for aerobactin in *E. coli CFT073*

In UPEC, the aerobactin iron uptake system has been shown to contribute to serum resistance as well as to bacterial survival and growth in the host (51). IroN, a novel catecholate siderophore receptor, has been shown to be more prevalent in *E. coli* isolates from UTI or bacteremia specimens than in fecal *E. coli* isolates (287).

The majority of APEC strains possesses the genes *iutA* and *iucC* and produced aerobactin relative to non-pathogenic avian isolates (86.3% vs 7.8% (183), 92.3% vs 63.5% (234), 63% vs 12% (73)). The deletion of this operon led to a decrease of the persistence of bacteria in the blood and also reduced damage of the respiratory tract and deep organs within a reproduction model of the avian colibacillosis on chicken (81). The same authors have identified the *iroN* gene coding for the siderophore receptor a counterpart of the UPEC strain 536 *iroN* gene (308). This gene is part of an operon consisting of the five genes *iroBCDEN* coding for the salmochelin system, which is also encoded by the plasmid ColV (160). The inactivation of the *iro* operon reduced the persistence of the bacterium in the blood and the respiratory tract lesions and deep organs indicating that this group of genes is required for the pathogenesis of avian colibacillosis (54, 81). Among *E. coli* isolates, *iro* sequences were shown to be associated with ExPEC isolated from neonatal meningitis (232), UTI, and prostatitis in humans (23, 163, 287) as well as APEC (99, 281).

Several authors have identified the presence of an additional siderophore: yersiniabactin encoded on the high pathogenicity island (HPI) of pathogenic yersiniae (165, 297, 298). The *irp1* and *irp2* genes encode proteins likely to be involved in the production of yersiniabactin and *fuyA* code for the receptor for yersiniabactin FyuA (106, 253). These genes have been detected in 89% of septicaemic APEC strains of serogroup O78 and in 80% of O2 strains (115). It is also interesting to note that 70% of these isolates (serogroup O78 and O2 combined) carry the *iucD* gene of the aerobactin operon suggesting that these two iron acquisition systems exist independently of one another among many isolates *E. coli* (115). More recently, two other siderophore systems, i.e. the Sit (*Salmonella* iron transport), a member of the Mn-Zn-Fe transporter family, and Chu (*E. coli* haeme utilization) system were highlighted by several authors, their involvement in the virulence of APEC has not yet been identified (196, 221, 289, 296, 313).

The homologous SitABCD system of APEC strain χ7122, encoded by the plasmid pAPEC-1 can transport both, Fe^{2+}, Fe^{3+} and Mn^{2+} ions, characteristic of carriers of the Mn-Zn-Fe transporter family. The isogenic *sitABCD* deletion mutant of the APEC strain χ7122 was tested in an experimental reproduction model of the colibacillosis on chicken. The severity of injuries caused in chickens has been the same for the mutated strain and to the wild-type strain. These results suggest that the absence of the SitABCD system is probably compensated by the presence of other iron or metals acquisition systems (289). It may be hypothesized that these systems are likely to have a role in growth of the bacteria at different stages of the avian colibacillosis, though their exact role in the infection process remains poorly understood and despite the high prevalence of these systems among APEC.

In UPEC, Chu system is involved in formation of intracellular bacterial communities (IBCs) in bladder urothelial cells but not the Sit system (275). Iha is expressed *in vivo* in the mouse urinary tract and functions as a catecholate siderophore receptor (195).

2.3.5 Phosphate transporter system (Pst)

The Pst system is a phosphate ATP-dependent transporter, a family member of the ABC-transporters (for a review Higgins, 2001). The corresponding proteins are encoded by the operon *pstSCAB-phoU* which is located on the chromosome and belongs to the Pho regulon (270) (Figure 9). This transporter is a system for inorganic phosphate (Pi) acquisition in a reduced Pi environment, a cellular component important for phosphorylation of nucleic acids, lipids, sugars and proteins (327).

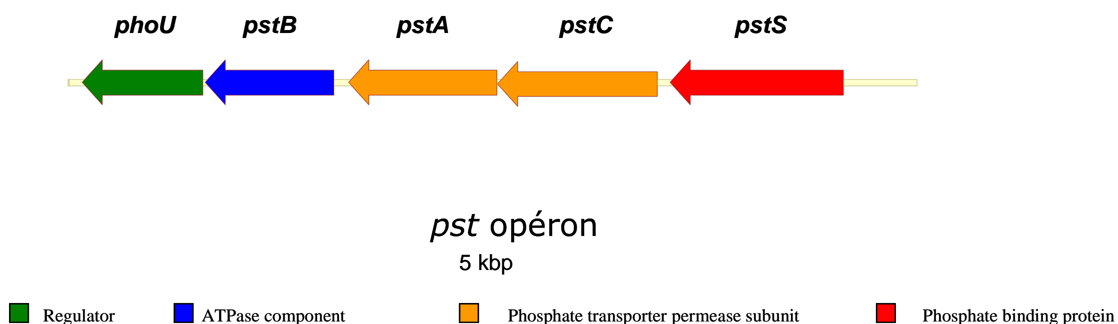


Figure 9: Organisation of the *pst-phoU* operon coding for the Pst system in *E. coli* CFT073.

The PstS protein is periplasmic, PstA and PstC are transmembrane and PstB binds ATP (8, 318). PhoU probably represses the expression of the Pho regulon in the presence of high concentrations of phosphate (347). A mutation of the *pst-phoU* operon triggered constitutive expression of the Pho regulon in a porcine septicaemic ExPEC strain and reduced its pathogenicity (66).

STM (signature-tagged mutagenesis) methods with insertions in different sites of the *phoU* gene are attenuated in the ability to cause UTI. These mutants were outcompeted approximately 100-fold by the wild-type strain at all sites in the urinary tract (19). Recently, it has been demonstrated with the *phoU* mutant and its complemented variant that PhoU contributes to efficient colonization of the murine urinary tract and thus PhoU was added to the list of confirmed urovirulence factors (47).

The APEC strain χ7122 has virulence characteristics common to a porcine SEPEC strain such as the ability to withstand the effects of serum (66, 82). In view of these similarities, Lamarche *et al.* have studied the implication of the *pst-phoU* operon in

virulence of the APEC strain χ7122 to the chicken (184). Thus, the strain deleted of the *pstCAB* operon formed significantly less lesions in the host, lost its ability to multiply in the blood and to colonize extraintestinal organs analysed (lungs and spleen). The mutant exhibited phenotypic changes (heightened sensitivity to the acid shock and polymixin) indicating a likely deterioration of the composition of the bacterial surface, which could partly explain the decrease in virulence (184). Moreover, this operon seems not to be involved in the resistance to bactericidal chicken serum. This indicates that the *pst* operon is not involved in the early stages of respiratory colibacillosis (184).

2.3.6 The Tsh protein and other autotransporters

Autotransporter proteins are also widely distributed in *E. coli* (138). The autotransporteurs are high molecular weight proteins organized into several functional domains. The thermosensible-hemagglutinin (Tsh) is part of the serine protease autotransporters of *Enterobacteriaceae* family (SPATE). Tsh exhibits similarity in its secretion mechanism with IgA (Immunoglobulin A) and with serine proteases of *Neisseria gonorrhoeae* and *Haemophilus influenzae* (266). The Tsh protein is composed of two parts, the secreted domain TshS and the Tshβ domain anchored in the outer membrane (312). The gene encoding the Tsh protein is located on the plasmid ColV near the cluster of genes coding for colicine V (82). However, the gene has been identified by hybridization to be chromosomally located as well in an APEC strain (44). In the study conducted by Maurer *et al.* the *tsh* gene is present in 46% of APEC pathotype isolates tested but not in commensal strains (209), while other studies have shown a strong association of the *tsh* gene with commensal strains (39.5% vs 3.8% (74), 90.4% vs 51.9% (234)). Dozois *et al.* confirmed the association of *tsh* to the APEC showing a lethality test on one day chicks. Results indicated that among *tsh*-positive APEC strains (46.2 % of tested strains) 90 % belong to the class of high lethality (LC1) (82). These same authors show that presence of the *tsh* gene is associated with the amount of fibrin and lesion development in the air sacs but not in other targeted sites of the colibacillosis (82). This suggests the role of Tsh as a putative adhesin in colonization of the air sacs but not in the development of systemic infection. So far, the potential role

of Tsh in the avian colibacillosis results from the more frequent association of the encoding gene with APEC strains than with non-pathogenic avian strains. The protease function deducible from the Tsh structure has not yet been demonstrated. However, a recent study has highlighted the adhesive and proteolytic properties of Tsh (182). The authors have shown that the purified protein TshS not only adheres to red blood cells but also to hemoglobin and the proteins of the extracellular matrix (fibronectin and collagen type IV). The proteolytic activity of TshS against casein has also been published (182). Further studies are needed to determine the ro1e of the proteolytic activity in the virulence of APEC strains.

Heimer *et al*. demonstrated that the autotransporter-encoding genes *pic* (SPATE homologue) and *tsh* are associated with *E. coli* strains that cause acute pyelonephritis and are expressed during urinary tract infection. These determinants have been found more frequently in UPEC strains than in fecal *E. coli*, suggesting a role in virulence (135).

Other SPATEs are considered to be toxins as well: the plasmid-encoded toxin (Pet) of EAEC, the protease Pic of EAEC and *Shigella flexneri,* EspC of EPEC, EspP of EHEC, SepA of *Shigella flexneri,* and Sat of UPEC (86). The Sat (Secreted Autotransporter Toxin) protein is widely distributed in UPEC and was shown to have cytopathic activity (elongation and vacuolation of eukaryotic cells). Sat-specific antibodies were found in the serum of *E. coli*-infected mice. Nevertheless the inactivation of the *sat* gene did not attenuate the *E. coli* strain CFT073 (124). All SPATEs possess a characteristic GDSGS serine protease motif and it is tempting to speculate that their protease activity may serve as peptide-providing source for the bacteria.

2.3.7 Complement resistance

The complement system is one of the early stages of host defense against micro-organisms. The ability to withstand the effects of complement is essentially due to the K1 capsule, certain outer membrane proteins (Omp) in the outer membrane or other proteins such as Iss (Increased serum survival), or the lipopolysaccharides.

2.3.7.1 The K1 capsule

The *E. coli* capsular antigen Kl is known to be an essential virulence factor of neonatal meningitis strains, with 80% of K1 capsule-positive *E. coli* strains isolated from neonatal septicaemia or acute pyelonephritis (175). The K1 capsule consists of a linear homopolymer of N-acetytneuraminic acid (NeuNAc). The biosynthesis and transport of the *E. coli* Kl capsule are mediated by a polycistronic region of 17 kb located on the chromosome which is divided into three functional regions (358) (Figure 10). The region 2 (encoded by the genes *neuUDEBACED* (34)) is unique to each K antigen and codes for the proteins involved in the synthesis, activation and polymerization of sialic acid. The region 1 contains two genes (*kpsMT*); the region 3 is composed of six genes (*kpsFEDUCS*). The latter two regions are highly conserved across the species *E. coli*. These genes are required for the transport of the capsular polysaccharides through the cytoplasmic membrane (KpsM and KpsT) and their assembly on the surface of the bacterium (KpsD and KpsE) (358).

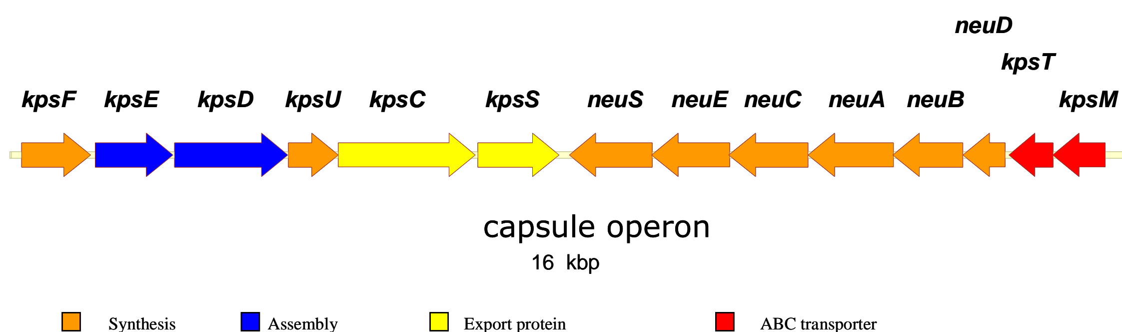


Figure 10: Organisation of the polycistronic *kps* region coding for the K1 capsule in *E. coli*

The Kl antigen is frequently associated with of the most represented APEC serogroups O1 and O2 (77, 119). The immunogenic properties of this surface antigen are weak and could therefore be associated with the APEC resistance to the bird immune defense system. Kl capsule-positive APEC are more resistant to serum than non-encapsulated *E. coli* strains (259). Mellata *et al.* have confirmed the results obtained by Pourbakhsh *et al.* by comparing the ability of a spontaneous non-encapsulated mutant and its wild-type Kl capsule-positive variant to resist to the effects of complement and have

also shown the importance of this surface antigen in the association of APEC strains to phagocytes (215, 216).

2.3.7.2 The outer membrane proteins OmpA, TraT and Iss

The outer membrane proteins (Omp) belong to at least two types: structural proteins and porins permit the passage of small molecules (235). Three proteins of the outer membrane, OmpA, TraT and Iss, play a more or less important role in the resistance to serum. The first studies on the role of OmpA in the *in vivo* pathogenesis of *E. coli* for chicken were conducted in 1991 (352). By the comparison of a Kl capsule- and OmpA-positive *E. coli* strain with its *ompA* mutant, the authors demonstrated the role of the OmpA protein in resistance to serum *in vitro* and *in vivo* as well as its ro1e as a virulence factor in chicken (263, 352).

The *traT* gene is carried on conjugative plasmids such as plasmid R6-5 of *E. coli* or plasmid ColV (3, 349). The lipoprotein TraT causes a structural and/or functional change of complement proteins which reduces their interactions with proteins on the bacterial surface. Accordingly, phagocytosis of bacteria is reduced in serum (3). Unlike for *ompA*, no correlation has been established between the presence of *traT* and virulence as measured by a fatality test on chicken embryos (361). A prevalence study of the *traT* gene showed a distribution equivalent of this gene among APEC strains and non-pathogenic strains. Thus, the authors assigned TraT a minor role in APEC pathogenesis (254). However, the genes *traT*, *traK* and *traG* are expressed *in vivo* during chicken infection (81).

The *iss* gene is carried on the ColV plasmid and codes for a lipoprotein of the outer membrane (60). This gene is mainly present in isolates associated with avian colibacillosis when compared with isolates from healthy animals (76.6% vs 18.7% (254), 82.7% vs 18.3%(281)). The relationship between the presence of the *iss* gene and the complement resistance was first established by Binns *et al.* (31). These authors observed that when the *iss* gene was introduced *in trans* into an *E. coli* K-12 strain, resistance to

bactericidal serum was increased (31). Moreover, a strain deleted for the Kl capsule determinant was more sensitive to complement than an *iss* mutant, suggesting that Iss play a less important role for serum resistance than the K1 capsule (324).

2.3.7.3 The lipopolysaccharide complex

Lipopolysaccharide (LPS) is a key component of the outer membrane of Gram-negative bacteria. It comprises three distinct regions: Lipid A, the oligosaccharide core, and commonly a long-chain polysaccharide, the O side chain that causes a smooth phenotype (see Figure 11).

Lipid A is the most conserved part of LPS. It is connected to the core part, which links it to the O repeating units (Figure 11). In *E. coli*, five different core structures (K-12 and R1-R4) have been described (9, 144, 277). The O repeating units are highly polymorphic, and more than 190 serologically distinguished forms in *E. coli* are known today (136, 243). The genes coding for LPS core synthesis are located at a conserved position on the *E. coli* K-12 chromosomal map (81-82 min) (26). The *wa** (formerly *rfa*) gene clusters contain the genes which code for the enzymes required for the core biosynthesis and assembly and consist of three operons, defined by their first genes *gmhD, waaQ* and *waaA*. Although the O repeating unit-encoding gene cluster (*wb**, former *rfb*) is extremely polymorphic within the species *E. coli*, it is localized at a conserved position on the *E. coli* K-12 chromosome between the genes *galF* and *gnd* (45.4 min.) (26).

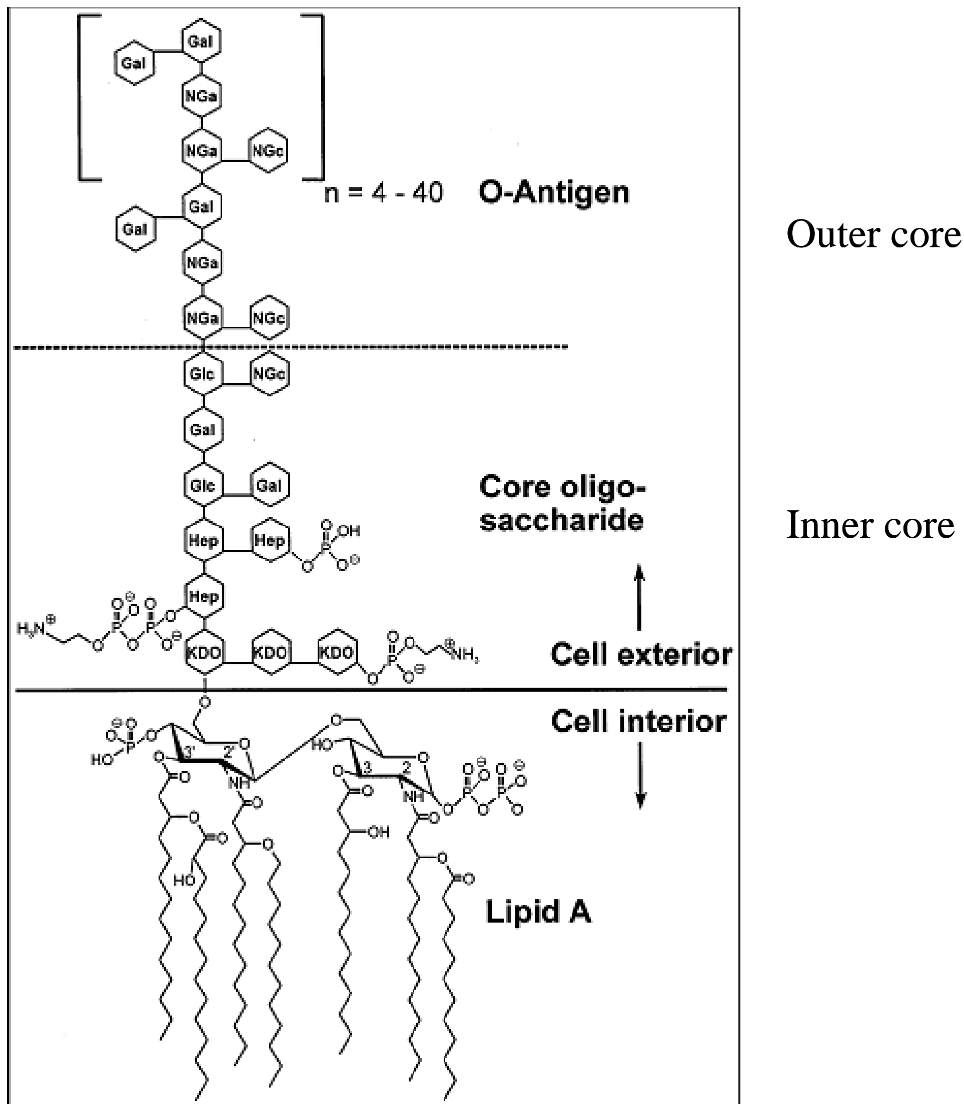


Figure 11: Chemical structure of LPS from *E. coli* O111:B4 according to Ohno and Morrison 1989 (241). (Hep) L-glycerol-D-manno-heptose; (Gal) galactose; (Glc) glucose; (KDO) 2-keto-3-deoxyoctonic acid; (NGa) N-acetyl-galactosamine; (NGc) N-acetyl-glucosamine.

These determinants consist of several sugar transferase-, epimerase- and isomerase-encoding genes, the O antigen flippase (*wzx*), the O antigen polymerase (*wzy*, formerly *rfc*) as well as the genes coding for enzymes involved in carbohydrate biosynthesis pathways. Until now, several *E. coli* O antigen-encoding gene clusters have been studied, e.g. those of serotypes O7, O111, O113, and O157 (206, 250, 345, 346). They show no significant nucleotide homology between each other, with the exception of some common genes such as *manC* and *manB*. However, they contain a conserved range

of predicted enzyme activities. The O6 antigen is widely distributed among pathogenic and non-pathogenic faecal *E. coli* isolates and is often found in uropathogenic *E. coli* strains. Since LPS is located on the outer surface of bacterial cells, its expression is known to be responsible for many features of the cell surface of the Gram–negative bacteria, such as resistance to detergents, hydrophobic antibiotics, organic acids, serum complement factors, adherence to eukaryotic cells (22, 112, 120, 152, 199, 320). It has been suggested that some of these characteristics, especially resistance to the bactericidal effect of the complement system, are dependent on the length of the O side chain (257). LPS is believed to significantly contribute to virulence by protecting bacteria from the bactericidal effect of serum complement (150, 273, 335). Moreover, it has recently been reported that the K5 capsule does not contribute as much to serum resistance of *E. coli* strains as the O antigen (50). The lipid A is endowed with toxic properties and represents the endotoxin of Gram-negative bacteria that can be released only upon bacterial lysis. The synthesis of several types of extracellular polysaccharides is necessary for optimal urovirulence (19).

Ellis and co-workers studied the relationship between serum resistance and virulence of pathogenic *E. coli* strains isolated from turkeys and showed a correlation between the serogroup of the strains and resistance to serum (93). Eight of the ten APEC strains tested showed resistance to the effects of complement. Among these eight strains, seven belonged to serogroup O78. The *rfb* locus coding for the LPS, is expressed *in vivo* during infection indicating its importance for APEC virulence (81). By comparing a wild-type pathogenic smooth O78 strain and its O78 O-side chain mutant, the authors have documented the importance of this surface antigen for bacterias resistance to host defenses. The O78-mutant, unlike the wild-type, could not resist to the bactericidal effect of serum. Moreover, in an experimental reproduction model of avian colibacillosis, the O78 LPS-mutant exhibited reduced virulence features compared to its $O78^{+}$ wild type strain (216). These studies demonstrate the importance of the surface antigen O78 for the virulence of APEC.

2.3.8 Toxins and bacteriocins

Toxins are prominent virulence factors of bacterial pathogens. Three toxins play a major role during UTI: the cytotoxic necrotizing factor 1 (CNF 1), the cytolethal distending toxin-1 (CDT-1) and α-haemolysin. CNF 1 is widely distributed in extraintestinal pathogens (12) and belongs to a toxin family which modifies Rho, a subfamily of small GTP-binding proteins that are regulators of the actin cytoskeleton (6). The gene for CNF 1 is chromosomally located on different pathogenicity islands of UPEC (36, 328). Eukaryotic cells intoxicated with CNF 1 exhibit membrane ruffling, formation of focal adhesions and actin stress fibers and DNA replication in absence of cell division.

CDT-1 is a secreted protein which has the capacity to inhibit cellular proliferation by inducing an irreversible cell cycle block at the G2/M position (61). CDT-1 is composed of three polypeptides (CdtA, B and C) which are all required for CDT activity (94). The direct role of the toxin in uroinfection, however, remains to be proven. The α-haemolysin is a member of the RTX toxin family, which is widely disseminated among pathogenic bacteria and widely distributed in UPEC as well as in EHEC isolates. The *hly* gene cluster encoding the toxin and the enzymes for its biosynthesis is located on PAIs or on plasmids. The type I secretion pathway, a posttranslational maturation and the presence of C-terminal calcium binding domain are characteristics of this pore-forming toxin (143, 344).

Other secreted compounds, such as colicins and microcins, are also widespread among *E. coli* strains and are believed to mediate antagonistic relationships, thus contributing to competitiveness and the effective colonization of the host. Microcins are peptides of a relatively small size (1.18 to 9.00 kDa). They are considered as modified peptide antibiotics since they are synthesized as peptide precursors which are subsequently modified by other proteins. They recognize a wide range of cellular targets: colicin B17 has been shown to be an inhibitor of the DNA gyrase (343), colicin C7 inhibits protein synthesis (121), and colicin V disrupts the membrane potential (366).

Microcin H47, encoded by the chromosomally-located *mch* gene cluster, was shown to be ribosomally synthesized as a peptide precursor (280).

APEC strains rarely produce toxins (32, 77). Two types of toxic effects were observed in 22.5% of APEC strains isolated from septicaemic chickens and turkeys in a study of 500 strains: a cytotoxic effect on Vero cells and/or on Y-1 cells culture (95). However, Fantinatti *et al.* observed a cytotoxic effect on Vero cells for only three strains isolated from septicaemic chickens on 17 (101). Some studies show that a small proportion of avian strains possess the genes encoding Shiga toxins Stx1 and Stx2, CNF 1 or CNF 2 toxins (32, 65, 73). Salvadori *et al.* have highlighted a cytotoxic effect of certain APEC strains on several avian cell lines in culture (kidney cells and fibroblasts), which is manifested by the formation of vacuoles (290). The toxin, called Vat for vacuolating autotransporter toxin, is a new member of the family SPATE (138, 247). The gene *vat* was located on a pathogenicity island recently identified in APEC strain Ec222 (249). A Δ*vat* isogenic mutant strain of the Ec222 was tested in two models of infection and revealed to be non-virulent for chickens (249). It is still not deciphered today if the cell and tissue damage of the host during the infection process of avian colibacillosis is due to the direct action of these toxins or simply the activation of an inflammatory response of the host.

2.4 Potential other virulence factors

In case of the APEC, all virulence factors described above are not sufficient to explain all stages of the pathogenesis of respiratory colibacillosis and their role is not clearly defined. The recent identification of new potential virulence factors of APEC could provide new data for a better understanding of their role in respiratory colibacillosis.

Among these new virulence factors we can cite the Fl7 fimbriae. These fimbriae were mainly reported in pathogenic *E. coli* strains responsible for diarrhoea or septicaemia in cattle and sheep (189, 197, 244). It was only recently that these adhesins

have been associated with APEC (316). Few APEC strains express these fimbriae. A recent study conducted on 763 strains APEC and 208 non-pathogenic avian strains found that only 7.7% of APEC strains have the *f17A* gene. However, only one non-pathogenic strain (0.48%) proved to be positive for this gene (316). The pathogenicity of certain strains with the F17 adhesin but without P-fimbriae has been tested in an experimental model of the avian colibacillosis. Fl7-positive APEC strains were pathogenic for chicken and caused characteristic lesions of avian colibacillosis (315). However, these results are not sufficient to conclude that F17 fimbriae are a virulence factor of APEC, because the strains possess other known potential virulence factors: fimbriae type I and/or iron acquisition system and/or Tsh protein (315). F17-related adhesins were also identified in human UPEC and bovine septicaemic *E. coli* strains (207).

Mat fimbriae is a novel fimbrial type recently detected in human O18:K1:H7 strain IHE3034 (262). The binding specificity or the function of these fimbriae is not known. The 7-kb *mat* region required for Mat fimbriae expression does not share significant homology with DNA encoding other filamentous adhesins. The *mat* gene cluster is found to be highly homologous (97-99 %) in *Klebsiella pneumoniae* and in various *E. coli* pathogroups but it seems to be absent in ETEC strains. Expression of Mat fimbria is probably differently regulated in various *E. coli* pathogroups as it was detected only in MENEC strains *in vitro*. *Rendon et al.* demonstrated its expression in intestinal commensal and pathogenic *E. coli*, in an *in vitro* model. Isogenic mutants of EHEC O157:H7 or of commensal Mat fimbriae-negative *E. coli* showed significant reduction in adherence to cultured epithelial cells. This pilus may have a potential role in host epithelial cell colonization and may represent an adherence factor of both pathogenic and commensal *E. coli* (276).

Different studies on virulence factors in septicemic *E. coli* strains of various hosts like humans, birds and lambs (220) show a variable profile of virulence genes as well as the presence of mobility-related sequences, indicating the existence of a ‘mix-and-match’ combinatorial system of virulence factors that can be used. The important conclusion of this study is that the distribution of virulence factors was independent of the host: bacteria

from the same host can have different virulence-related genes, just as bacteria from different hosts can share the same virulence-related sequences.

A recent study on APEC, UPEC, and NMEC, based on serotyping, virulence genotyping, and ECOR grouping (99) supports the hypotheses that poultry may be a vehicle or even a reservoir for human ExPEC strains, that APEC potentially serve as a reservoir for virulence-associated genes of UPEC and NMEC, and that some ExPEC strains, although of different pathotypes, may share common ancestors.

Several molecular epidemiological studies by multilocus sequence typing (MLST), which is highly discriminatory to analyze clonal relationships, support the results from subtractive hybridization and DNA sequencing. They show that the profile of virulence factors in ExPEC strains, as well as their clonal relationship, is independent of the host and independent of the serotype. However, there may be some degree of host specificity in ExPEC strains, because *E. coli* strains isolated from avian septicemia are more virulent to chicks than *E. coli* strains isolated from new born meningitis (285).

Recent studies about the comparison of sequences of avian and human extraintestinal pathogenic *E. coli* by genomic subtractive hybridization (167) or genome sequencing of a representative APEC strain (159) underline the genetic variability of ExPEC as well as genomic similarities between UPEC and APEC. Nevertheless, they did not identify any single marker that would determine host and/or niche specificity of APEC or UPEC.

2.5 Aims of this study

As specific virulence factors that trigger the infection of either humans or poultry could not be identified yet, one aim of this study was to investigate whether differential regulation of virulence gene expression may be responsible for host specificity of APEC and human ExPEC strains. A thorough analysis of factors that contribute to the host specificity (human vs. poultry) of ExPEC strains is therefore important to evaluate the

zoonotic risk emerging from these bacteria. In this context, the difference between the human and the avian body temperature (37 °C and 41-42 °C, respectively) could be important. Consequently, the transcriptome of a human and an avian ExPEC isolate of serotype O18:K1 in response to different growth temperatures should be compared by DNA array hybridization to identify genes that are specifically transcribed either in response to the body temperature of humans (37 °C) and avians (41 °C) or depending on the strain background.

In the second part of my thesis, the expression of Mat fimbriae of MENEC, O18:K1:H7 strain IHE3034 was investigated. The role of the *matA* gene, coding for the putative regulator of Mat fimbriae, was studied by transcriptome comparison of the wild type and its isogenic *matA* mutant, carrying a mutation in the helix-turn-helix domain of the protein. Additionally, the genetic organization of the *mat* gene cluster was studied, the *matA* promoter was analysed and its transcription start point was determined. The influence of different growth temperatures and transcription factors on *mat* gene expression was analysed as well. The results should improve our knowledge on the molecular mechanisms underlying different gene expression patterns in different ExPEC strains.

3. Material

3.1 Strains

All bacterial strains used in this study and their relevant genotype are listed in Table 4.

Table 4: Bacterial strains used in this study.

Strain	Relevant properties	Reference
E. coli MG1655	OR:K$^-$:H48, F$^-$, λ$^-$, *ilvG*, *rfb*-50 *rph*-1	(Blattner et al., 1997)
E. coli DH5α	F-, *end*A1, *hsd*R17 (r_{k-}, m_{k-}), *sup*E44, *thi*-1, *rec*A1, *gyrA*96, *rel*A1, λ$^-$, Δ(*argF-lac*)U169, Φ80*dlac*Z ΔM15	(Bethesda Research Laboratories)
E. coli IHE3034	ExPEC O18:K1:H7, newborn meningitis isolate	(181)
E. coli BEN374	ExPEC O18:K1:H7, avian septicaemia isolate.	(M. Moulin-Schouleur, INRA Centre de Tours, France)
E. coli RS218	ExPEC O18:K1: H7, newborn mengitis isolate.	(352)
E. coli BEN79	ExPEC O18:K1:H7, avian septicaemia isolate.	(M. Moulin-Schouleur, INRA Centre de Tours, France)
E. coli IHE3072	ExPEC O2:K1: H5, newborn meningitis isolate.	(Achtman et al., 1986)
E. coli BEN2908	ExPEC O2:K1:H7, avian septicaemia isolate.	(Schouler et al., 2004)
E. coli 1772	ExPEC O2:K1, avian septicaemia isolate.	(Mokady et al., 2005)
E. coli 285	ExPEC O78, newborn meningitis isolate.	(Mokady et al., 2005)
E. coli 789	ExPEC O78, avian septicaemia isolate.	(Yerushalmi et al., 1990)
IHE3034*Δhcha*	chromosomal deletions of CDS	(this study)
IHE3034*ΔmatA*	chromosomal deletions of CDS	(this study)
BEN374*ΔmatA*	chromosomal deletions of CDS	(this study)
BEN374*ΔmatB*	chromosomal deletions of CDS	(this study)
MG1655*ΔmatA*	chromosomal deletions of CDS	(this study)

3.2 Plasmids

All plasmids used and constructed during this study are listed in Table 5.

Table 5: Plasmids used in this study.

Plasmid	Relevant properties	Reference
pKD46	*repA101* (ts), *araBp-gam-bet-exo* (λred recombinase under the contol of *araB* promotor), ApR (*bla*)	(70)
pKD3	oriRg, ApR, *cat*-gene flanked by FRT sites	(70)
pKD4	oriRg, ApR, *npt*-gene flanked by FRT sites	(70)
pCP20	Yeast Flp recombinase gene (FLP, aka exo) *ts-rep*, ApR,CmR	(70)
pGEMT-*matA*		This study
pBAU1	gene fusion of MBP and MatA	This study

3.3 Medium

Bacteria were routinely grown in Luria-Bertani (LB) broth at 37 °C with shaking at 220 rpm or on LB agar plates if not state otherwise. Bacteria carrying temperature-sensitive plasmids (pKD46) were grown at permissive temperatures (30°C).

When appropriate, media were supplemented with chloramphenicol (20 µg/ml), kanamycin (30 µg/ml), or ampicillin (100 µg/ml).

All media were autoclaved for 20 min at 120 °C, if not stated otherwise. Supplements for media and plates were sterile filtered through a 0.22 µm pore-filter and added after cooling down the media to <50 °C.

3.3.1 Media

LB medium (Luria-Bertani): (Sambrook, 1989)

10 g tryptone from casein

5 g yeast extract

5 g NaCl ad 1 l dH_2O

LB agar plates:

LB medium + 1.5 % (w/v) agar (Difco Laboratories, Detroit, USA)

Motility agar plates:

LB medium + 0.3 % (w/v) agar

Blood agar plates:

LB plates containing 5 % (v/v) washed sheep erythrocytes

(Elocin Lab, Mühlheim a. d. Ruhr)

X-Gal medium:

LB-medium supplemented with the following additives:

IPTG (0.1 M)

X-Gal (2 %, (w/v) in N,N'-dimethylformamide)

Yeast-tryptone medium (YT):

Tryptone 16 g

Yeast extract 10 g

NaCl 10 g

dH_2O add 1000 ml

Medium for detection of aerobactin expression (Braun, Gross et al. 1983; Ott, Bender et al. 1991):

Aerobactin agar plates:	
Nutrient broth	4 g
NaCl	2.5 g
Agar	6 g
Dipyridyl (200 mM)	5 ml
Titriplex (10 mM)	5 ml
dH_2O	add 500 ml
Soft agar for aerobactin agar plates:	
Nutrient broth	0.8 g
NaCl	0.5 g
Agar	0.75 g
Dipyridyl 200 mM	1 ml
Titriplex 10 mM	1 ml
E. coli strain EN99 overnight culture (YT,Tc)	4 ml
dH_2O	add 100 ml

Congo red medium:

The congo red and Coomassie brilliant blue stock solutions were sterilized by filtration through a 0.22 µm sterile filter and added to the autoclaved medium.

Trypton 10 g

Congo-red (0.4 mg/ml) 1 ml

Coomassie brilliant blue (0.2 mg/ml) 1 ml

dH2O add 1000ml

M63B1 minimal medium:

10 ml 20 % (w/v) $(NH_4)_2SO_4$ solution

13.6 g KH_2PO_4

1 ml 10 % (w/v) $MgSO_4$ solution

1 ml 0.1 % (w/v) $FeSO_4$ solution ad 978 ml dH_2O

pH adjusted to 7 with KOH and autoclaved; then addition of

2 ml 0.05 % (w/v) thiamine (sterile filtered)

20 ml 20 % (w/v) glucose (sterile filtered)

CAS agar plates: (Schwyn and Neilands 1987)

I) *Basal agar medium*:

30.24 g PIPES; dissolved in 250 ml dH_2O + 12 ml 50 % NaOH

100 ml 10 x MM9 salts 3 g /l KH_2PO_4

5 g /l NaCl

10 g /l NH_4Cl

autoclaved and cooled to 50 °C;

15 g agar dissolved in 500 ml dH_2O

autoclaved, cooled to 50 °C and mixed with first solution; then addition of

30 ml 10 % (w/v) casamino acids solution (sterile filtered)

10 ml 20 % (w/v) glucose solution (sterile filtered)

II) *CAS indicator solution*: (100 ml)

60.5 mg Chrome azurole S (CAS; Sigma) dissolved in 50 ml dH_2O

10 ml iron III solution: 27 mg $FeCl_3$ x 6 H_2O

83.3 µl 37 % HCl

ad 100 ml dH_2O

To the above solution, 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA) dissolved in 40 ml distilled water was added slowly under stirring. The resultant dark blue solution was sterile filtered, heated to 50 °C and added very slowly along the glass walls to the basal agar medium before pouring approximately 25 ml into each plate.

3.3.2 Antibiotics

When appropriate, media and plates were supplemented with the antibiotics listed in Table 6 in the indicated concentrations. Stock solutions were sterile filtered and stored at -20 °C until usage.

Table 6: Antibiotic substances used in this study.

Antibiotic	Stock concentration	Solvent	Working concentration
Chloramphenicol (Cm)	50 mg/ml	EtOH	20 µg/ml
Ampicillin (Ap)	100 mg/ml	dH_2O	100 µg/ml
Kanamycin (Km)	50 mg/ml	dH_2O	50 µg/ml
anhydro-Tetracycline (aTc)	2 mg/ml	EtOH	0.2 µg/ml
Spectinomycin (Spec)	100 mg/ml	dH_2O	100 µg/ml

3.4 DNA and Protein Markers

To determine the size of DNA fragments in agarose gels, the “GenerulerTM” 1-kb DNA ladder, purchased from MBI Fermentas, was used. As a reference in denaturing agarosegels for Northern blotting, both the low-range RNA marker from Peqlab as well as the 0.24-9.5-kb RNA ladder from Invitrogen were used. To determine the molecular weight of protein fractions separated by polyacrylamide gel electrophoresis, “Rainbow marker” (RPN800) purchased from Amersham Biosciences was used.

3.5 Technical Equipment

Balances IL-180, Chyo Balance Corp

Kern 470

Ohaus Navigator

Autoclaves Integra Bioscience, H+P Varoclav

Bioanalyzer 2100 Agilent Techn., Palo Alto, CA

Incubators Memmert Tv40b (30 °C, 42 °C)

Heraeus B5050E (37 °C)

Clean bench NUAIRE, Class II, type A/B3

Electrophoresis systems BioRad

Electroporator Gene Pulser, BioRad

FPLC Amersham Pharmacia

Hybridization oven HybAid Mini 10

Centrifuges (cooled) Beckmann J2-HC ® JA10 and JA20 rotors

Haraeus Sepatech Megafuge1.OR

Haraeus Sepatech Biofuge 13R

Centrifuges (table top) Eppendorf 5415C

Hettich Mikro20

GenePix4000B microarray reader (GE Healthcare).

Magnetic stirrer Heidolf MR3001K

Micropipettes Eppendorf Research 0.5-10 µl

Gilson pipetman 20 µl, 200 µl, 1000 µl

Microwave AEG Micromat

Power supplies BioRad Power Pac 300

PCR-Thermocycler Biometra T3

pH-meter WTW pH 525

Documentation BioRad GelDoc2000 + MultiAnalyst Software V1.1

Developer Agfa Curix 60

Photometer Pharmacia Biotech Ultrospec 3000

Phosphoimager Amersham Biosciences, Typhoon 4600

Shakers Bühler TH30 SM-30 (37 °C, 150 rpm)

Innova 4300, New Brunswick Scientific (37 °C, 220 rpm)

Innova 4230, New Brunswick Scientific (30 °C, 220rpm)

Sonicator Bandelin Sonoplus HD70; Tip UW70

Speedvac Savant SC110

Thermoblocks Liebisch

Vacuum Blotter Pharmacia + LKD Vacu Gene Pump

Videoprinter Mitsubishi Hitachi, Cybertech Cb1

Vortexer Vortex-Genie 2TM Scientific Industries

UV-Crosslinker BioRad

Waterbath GFL 1083, Memmert

4. Methods

If not stated otherwise, all methods followed the instructions described in the CSH Laboratory Manual (Sambrook 1989). Centrifugations with no other indications were carried out in a table top centrifuge at 13,000 rpm.

4.1 Manipulation of DNA

4.1.1 Small scale isolation of plasmids

While using the QIAGEN Plasmid Midi and Mini Kit, bacteria were collected from 100 ml over night cultures by centrifugation (6000 rpm, 4 °C, 15 min) and resuspended in 4 ml buffer P1, according to the manufacturer's recommendations. After 5 min incubation at room temperature, 4 ml buffer P2 were added for lysis of the cells. After clearing of the suspension, 4 ml neutralization buffer P3 were added and samples were incubated for 10 min on ice. Cell debris and genomic DNA were removed by centrifugation (11000 rpm, 4 °C, 30 min). Plasmid DNA-containing supernatant was loaded on equilibrated columns by gravity flow. Columns were washed with buffer QC. Subsequently, plasmid DNA was eluted with 3.5 ml buffer QF and precipitated by addition of 0.7 vol isopropanol. After centrifugation (13000 rpm, 4 °C, 20 min), DNA pellets were washed with 70 % (v/v) ethanol, air-dried and resuspended in water.
Plasmid isolation using the QIAspin mini kit were performed similarly with some modifications: bacteria were harvested from 1-10 ml over night cultures, buffer N3 containing guanidine hydrochloride was used for neutralization, and plasmid DNA was purified from the supernatant by using small spin columns, which were centrifuged at 13000 rpm for 1 min. DNA was eluted in a small volume of dH_2O and directly used for further experiments.

4.1.2 Isolation of chromosomal DNA (Grimberg *et al.* 1987)

Bacteria from 1 ml of an over night culture were harvested by centrifugation for 4 min in a 1.5 ml reaction tube. After washing with 1 ml TNE buffer, cells were centrifuged for 4 min and resuspended in 270 µl TNE-X solution. 30 µl lysozyme (5 mg/ml) were added and samples were incubated for 20 min at 37 °C. Afterwards, 15 µl proteinase K (20 mg/ml) were added and further incubated for up to 2 h at 65 °C until the solution became clear. The genomic DNA was precipitated by addition of 0.05 vol 5 M NaCl (15 µl) and 500 µl ice-cold ethanol and then collected by centrifugation for 15 min. After washing two times with 1 ml 70 % (v/v) ethanol, DNA pellets were air-dried and redissolved in 100 µl sterile dH_2O.

TNE: 10 mM Tris
10 mM NaCl
10 mM EDTA pH 8.0
TNE-X: TNE + 1 % Triton X-100

4.1.3 Precipitation of DNA with alcohol

DNA was either precipitated with ethanol or with isopropanol. In the first case, 0.1 vol 3 M Na-acetate (pH 4.8) were added to the samples before the addition of 2.5 vol ice-cold 100 % (v/v) ethanol. For the precipitation with isopropanol, 0.7 vol were used. Samples were incubated for at least 1 h at -80 °C before centrifugation (13,000 rpm, 4 °C, >20 min). The DNA pellet was washed with 70 % (v/v) ethanol, dried in a Speedvac for 10 min and resuspended in dH_2O.

4.1.4 Determination of nucleic acid concentrations

Nucleic acid concentrations were measured at 260 nm in quartz cuvettes with a diameter of 1 cm. An absorption at 260 nm of 1.0 corresponds to 50 µg/ml double-stranded DNA or 40 µg/ml RNA. The purity of the preparations was determined by measuring the

absorption of the sample at 280 nm. DNA and RNA were considered sufficiently pure when the ratio A260 / A280 was higher than 1.8 or 2.0, respectively.

4.1.5 Polymerase chain reaction (PCR)

This method allows the exponential amplification of DNA regions *in vitro* by using a heat stable DNA polymerase from *Thermus aquaticus* (*Taq*). This way, even small amounts of template DNA can be amplified to high copy numbers and easily visualized during screening assays. Another application of PCR is site-directed mutagenesis by using oligonucleotides with adapted sequences, e.g. restriction sites.

Standard PCR

For routine PCR-amplification, *Taq* DNA polymerase kits of different suppliers (QIAGEN, Sigma) were used. Usually, the reaction was performed in a final volume of 20 µl.

Mix for one sample: 2 µl 10× reaction buffer (QIAGEN)
2 µl 20mM dNTP mix (Sigma)
0.6 µl 25 mM $MgCl_2$
0.2 µl 100 pM primer solution 1
0.2 µl 100 pM primer solution 2
1 µl 100 ng/µl template DNA or boiled cells
0.05 µl Taq DNA polymerase (QIAGEN)
14 µl dH_2O
For the Sigma Red Taq polymerase kit, both primers and template DNA were added to 8.6 µl
dH_2O and 10 µl 2× Red Taq ready mix (see the manufacturer's instructions).

The thermal profile was designed according to the annealing temperature of the individual primers and the length of the expected amplification product:
Initial denaturation 2 min, 95 °C

1. Denaturation 45 sec, 95 °C
2. Annealing 45 sec (54-60 °C)
3. Elongation 30 sec 0.5-5 min, 72 °C

(steps 1–3: 25-35 cycles)

Final elongation 2 min, 72 °C

PCR with proof-reading polymerases

For site-directed mutagenesis using PCR products, a different polymerase with 3'-5' proofreading activity had to be used in order to prevent misincorporations during extension. The composition of a typical PCR mix for the λ Red mutagenesis (see section 4.1.13) is given below:

Mix for 1 sample: 5 µl 10× Opti buffer (Eurogentec)
5 µl 20mM dNTP mix (Sigma)
3.5 µl /kb 50 mM $MgCl_2$
1 µl 100 pM primer solution 1
1 µl 100 pM primer solution 2
1 µl 100 ng/µl template plasmid DNA
0.5 µl DAP Goldstar polymerase (Eurogentec)
ad 50 µl dH_2O

4.1.6 Enzymatic digest of DNA with restriction endonucleases

The DNA was dissolved in dH_2O and mixed with 0.2 vol 10× reaction buffer and 1 U of restriction enzyme per µg DNA, so that the final volume of the sample was 15 µl for plasmid DNA and 50 µl for genomic DNA. The mixture was incubated at 37 °C, depending of the specific requirements of the enzyme indicated on the product sheets. Whereas plasmid DNA was digested for one to two hours, digestion of genomic DNA was carried out over night. The reaction was stopped by adding 0.2 vol stop-mix (see following section 4.1.7.). When appropriate, inactivation of the restriction enzyme was carried out by heating the samples for 20 min at 65 °C.

4.1.7 Horizontal Gel Electrophoresis

For routine analytical and preparative separation of DNA fragments, horizontal gel electrophoresis was performed using agarose gels under non-denaturing conditions. Depending on the size of the DNA fragments to be separated, the agarose concentration varied between 1 and 2 % (w/v) in running buffer (1× TAE). In order to have a visible running front and to prevent diffusion of the DNA, 0.2 vol loading dye was added to the samples before loading. The electrophoresis was carried out at a voltage in the range between 16-120 V. The gels were stained in an ethidium bromide solution (10 mg/ml), washed with water and photographed under a UV-transilluminator.

4.1.8 Isolation of DNA fragments from agarose gels

DNA was purified from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN). Agarose pieces containing the DNA fragment of interest were cut out of the gel and subsequently melted for 10 min at 50 °C in QG buffer (supplied by the manufacturer). The DNA was separated from the rest of the solution by applying the mixture to QIAquick spin columns followed by centrifugation for 1 min. The columns were then washed with 750 µl PE buffer (supplemented with ethanol). Residual PE buffer was removed by centrifugation (2× 1 min). Finally, the DNA was eluted from the column with 20-50 µl sterile dH_2O.

4.1.9 Ligation of DNA fragments

Linearized vector and insert DNA after restriction digest can be ligated either due to the presence of sticky ends or by blunt-end ligation. The modifying enzyme for ligation process was a T4-DNA ligase (New England Biolabs). Best efficiencies were obtained using a insert/vector ratio of 3/1. Reactions were performed over night at 16 °C in a final volume of 15 µl containing 1.5 µl 10× ligation buffer and 50 U T4 ligase.

4.1.10 Transformation of bacterial cells

Preparation of electrocompetent cells and electroporation

50 ml LB medium were inoculated with 500 µl of an over night culture of the strain of interest and grown OD_{600} of 0.6-0.8. The cells were collected by centrifugation for 10 min 6000 × g at 4 °C. The pellet was left on ice for 30 min and then washed with 50 ml ice-cold dH_2O. After a second centrifugation step at the same conditions, the pellet was resuspended in 25 ml 10 % (v/v) glycerol, centrifuged again and finally resuspended in 600 µl 10 % glycerol. The cells were stored as 40 µl aliquots at -80 °C. For electroporation, one aliquot was thawed on ice and mixed with ~ 0.5 µg DNA. The mixture was applied into a “Gene pulser” cuvette (BioRad) with a distance between the electrodes of 0.1 cm and incubated for 10 min on ice. The cells were electroporated using a Gene pulser transfection apparatus (BioRad) at the following conditions: 2.5 kV, 25 µF, and 600 Ω for linear fragments or 200 Ω for plasmids. Immediately after electroporation, 1 ml LB medium was added to the cuvettes. The mixture was transferred into a new tube and incubated at 37 °C (or 30 °C for temperature-sensitive plasmids) for 1 h before the cells were plated on selective agar plates.

4.1.11 A/T cloning of PCR products using the pGEM-T® Easy vector system

This kit enables rapid cloning of PCR fragments without digestion by overhanging adenine nucleotides at their 3' ends into a linearized vector that contains overhanging 5' terminal thymidine residues. pGEM-T® Easy vector allows cloning of DNA fragments in a multiple cloning site which is flanked by T7 and SP6 RNA polymerase promoters, respectively. This vector expresses the α-peptide of the β-galactosidase, thus enabling “blue-white” screening of successful DNA integration. The ligation reaction was performed overnight at 4 °C or for 2 h at RT and was prepared as follows:

2× T4 DNA ligation buffer 10 µl
pGEM-T® Easy vector 1 µl
PCR product 1-8 µl

T4 DNA ligase 1 µl
dH2O to final volume of 20 µl

4.1.12 Cloning of DNA fragments digested with restriction enzymes

DNA fragments digested with restriction enzymes were cloned in vectors that were cut with appropriate enzymes ("sticky ends ligation"). For ligation, digested vector and insert were mixed in a ratio of 1:3. The ligation reaction was performed overnight at 4 °C or 16 °C, or for 2 h at RT and was prepared as follows:
Linearized vector 0.5-1 µl
Restriction enzyme-digested DNA fragment 1-17.5 µl
5× T4 ligase buffer 2 µl
T4 ligase (New England BioLabs), 2 U/µl 1 µl
dH2O to final volume of 20 µl

When the vector and the insert were cut with only one restriction enzyme or for blunt end ligation, the ends of the linearized vector molecule were dephosphorylated before ligation using antarctic phosphatase (New England BioLabs) in order to prevent religation of the vector. Removal of the 5'-phosphate residue was carried out by addition of 0.1 vol 10× antarctic phosphatase reaction buffer ($ZnCl_2$ 1 mM; $MgCl_2$ 10 mM; Bis Tris-Propane 500 mM; pH 6.0; H2Obidest.), 1 U antarctic phosphatase and incubation for 1 h at 37 °C. The reaction was stopped by heating for 5 min at 65 °C.

4.1.13. Gene inactivation by λ Red recombinase-mediated mutagenesis using linear DNA fragments

The construction of the mutants was performed using linear DNA for recombination, as described by Wanner and Datsenko (2000). This method relies on the replacement of a chromosomal sequence with an antibiotic marker that is generated by PCR using primers with homology extensions to the flanking regions of the target sequence. Recombination is mediated by the Red recombinase derived from the λ phage. This recombination

system consists of three genes (*λ*, *ß*, *exo*), which encode the phage recombinases and an inhibitor of the host RecBCD exonuclease V, which normally mediates degradation of linear DNA in the cell. A schematic overview of the procedure is depicted in Figure 12. Briefly, a linear DNA fragment containing an antibiotic marker cassette flanked by FRT sites and 45-nt homologous extensions to the target genes (up- and downstream regions, respectively) were amplified by PCR as described in section 4.1.5. The proofreading Dap Goldstar polymerase (Eurogentec) was used for amplification of the linear fragments with plasmids pKD3 (harboring a chloramphenicol resistance cassette) or pKD4 (harboring a kanamycin resistance cassette) as template in a total volume of 400 µl. The annealing temperature for all primers was 54 °C using 30 cycles of amplification. PCR products were purified using the QIAquick PCR purification kit, ethanol precipitated and resuspended in 10 µl dH2O.

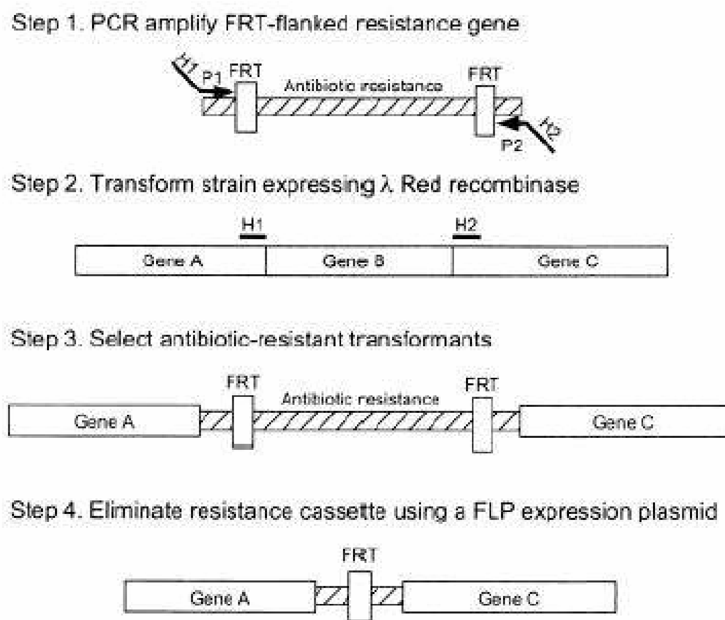


Figure 12: Strategy for inactivation of chromosomal genes using PCR products (70).

Meanwhile, the cells were first transformed with the pKD46 helper plasmid by electroporation (see section 4.1.10.). Transformants were selected at 30 °C on agar plates containing 100 µg/ml ampicillin. Of these transformants, electrocompetent cells were prepared from 50 ml LB cultures supplemented with ampicillin and 3 ml of an 0.1 M

arabinose solution to induce the Red recombinase on the helper plasmid. 40 µl competent cells were transformed with 5 µl of the linear PCR fragment by electroporation. After the addition of 1 ml LB medium to the cuvette, cells were incubated for 2 h at 30 °C with aeration. In contrast to normal electroporation, the cultures were then taken out of the incubator and left standing on the bench top over night at room temperature. In the next morning, cells were spun down, resuspended in 300 µl LB medium and distributed onto three agar plates supplemented with the appropriate antibiotic (Cm or Km, respectively). Transformants with confirmed allelic exchange were also re-streaked onto ampicillin-containing agar plates at 37 °C to confirm loss of the temperature-sensitive helper plasmid pKD46.

The antibiotic marker could be removed with the help of the FLP recombinase (encoded on plasmid pCP20), which mediates recombination between the two FRT sites flanking the antibiotic resistance cassette, thus leaving behind a complete deletion of the open reading frame.

Electroporation was performed as described in section 4.1.10.2. Transformants were first selected on ampicillin-containing agar plates at 30 °C, and then re-streaked onto LB agar plates with no antibiotic. These plates were incubated at 37 °C in order to induce the loss of the second helper plasmid pCP20. The deletion mutants now could be used to introduce a second or third mutation by starting the whole procedure from the beginning. All mutations were confirmed by both PCR and Southern hybridization.

Table 7: Primers use for gene deletions and control.

matA red direct stop	CCCGGTGAGTCATTTTTAAAACTAACTTGCCTGG AGTTTAGTGTAGGCTGGAGCTGCTTC
matA red reverse start	AGAAACTGAATGTACCTGTAAAAATTACAGGTT TGGAAAGTAGTGCATATGAATATCCTCCTTA
matA south left	TAATTTCCATTTCCCGGTCA
matA south right	CCCCATGACGCCTACTTCTA
matA apec start wanner left	CTGAATGTACCTGTAAAAATTACAGGTTTGGAAAGTAGTGG TGTAGGCTGGAGCTGCTTC
matA apec start wanner right	CCCGGTGAGTCATTTTTAAAACTAACTTGCCTGGAGTTTAA TGGGAATTAGCCATGGTCC
Primer vérif *matA* right	CTGTTCACATATTGACACTC
Primer verif *matA* left	GAGTTGAATTGAGGACATGA

Table 7: Primers use for gene deletions and control - continued

matB apec start wanner left	GACTTCATGTCCTCAATTCAACTCGGGAAGAAAAGCAATGAT TGTGTAGGCTGGAGCTGC
matB verif left	GGCCACTGTCGGTACTGTTT
matB verif right	CGCTGGACTGAGTCGTGATA
matB apec stop wanner right	AAAGCAGGGGGGTACCCCTGCTGGTACATCAGAGAGATTAATG GGAATTAGCCATGGTCC
yedU direct wanner start	ATAGTGACTACCCTAACTAAGCAACAATAAGGAATACACTATGG TGTAGGCTGGAGCTGCTTC
Wanner *yedU* reverse	GCGATTGATTATGCGCTTACATTCAAACGTAACAGGGATTAATG GGAATTAGCCATGGTCC
yedU verif left	TAGCGGCCAGCTCAGTCGCA
yedU verif right	CTGCGATTGATTATGCGCTT
cadA lambda red left	AAGGGAAGTGGCAAGCCACTTCCCTTGTACGAGCTAATTAGTG TAGGCTGGAGCTGCTTC
cadA lamda red right	AAAGTATTTTCCGAGGCTCCTCCTTTCATTTTGTCCCATGATGG GAATTAGCCATGGTCC
cadA verif left	GTGGCAAGCCACTTCCCTTG
cadA verif right	CGGTGAACTGACCGGTATCG

4.1.14 Southern Blot analysis

For Southern blot analysis, 10 µg chromosomal DNA were restricted with an appropriate endonuclease resulting in 1 to 5-kb DNA fragments with the target gene. The DNA fragments were separated by horizontal gel electrophoresis (see section 4.1.7.), denatured in a 0.5 M NaOH, 1.5 M NaCl solution for 30 min with shaking, and neutralized in a neutralization solution (0.5 M Tris-HCl, pH 7.5; 1.5M NaCl). Meanwhile, a nylon membrane (Nytran Super Charge; pore size 0.45 µm; Schleicher & Schuell, Dassel, Germany) of appropriate size was shortly pre-incubated in dH_2O and then soaked for 10 min in 20× SSC. Capillary blot was done overnight to transfer the DNA from the gel to the membrane.

After the DNA transfer, the nylon membrane was incubated for 1 min in 0.4 N NaOH and 1 min in 0.25 M Tris-HCl, pH 7.5 for neutralization. The membrane was then shortly dried and the DNA was crosslinkd to the membrane by exposure to UV light.

Probe labelling (ECL^{TM} Kit, Amersham Biosciences)

For labelling of DNA probes, the ECL^{TM}-Kit (enhanced chemoluminescence) was used. The binding of a DNA probe to the complementary sequence on the nylon membrane was detected by chemoluminescence. Positively charged horseradish peroxidase molecules

were mixed with the negatively charged DNA probe. Addition of glutaraldehyd covalently linked the horseradish peroxidase molecules with the DNA probes. Reduction of H_2O_2 by the peroxidase requires the oxidation of luminol which results in light emission, which can be detected by suitable light-sensitive films, e.g. the Hyperfilm ECL.

For labeling of the probe, 100 ng DNA per ml hybridization buffer in a final volume of 10 µl in dH_2O were denatured for 10 min at 90 °C and cooled for 5 min on ice. Subsequently, 10 µl labelling reagent and 10 µl glutaraldehyd were added. The mixture was incubated for 10 min at 37 °C and then added to the hybridization reaction.

Hybridization and detection of the membrane

Hybridization of the membrane was carried out over night at 42 °C in hybridization solution (10-15 ml), after the nylon membrane was pre-incubated at 42 °C in the hybridization solution for 1 h. The next day, the membrane was washed twice for 20 min at 55 °C with wash solution I and two times for 10 min at RT with wash solution II. The membrane was placed on Whatman paper to remove the rest of the wash solution, and then incubated for 5 min in 5-10 ml detection solution I and detection solution II provided with the kit and mixed immediately (1:1) before addition to the membrane. The membrane was superficially dried on Whatman paper and packed in saran wrap avoiding air bubbles on the top surface of the membrane. Chemoluminescence was detected by exposure of the membrane to Hyperfilm ECL. The exposure time depended on the signal intensity.

Wash solution 1: 0.5× SSC; 0.4 % (w/v) SDS

Wash solution 2: 2× SSC

4.1.15 Sequence analysis

The nucleotide sequences of mutagenized chromosomal genes or plasmid constructs were determined using fluorescent dye terminators (ABI prism BigDye terminator kit, Applied Biosystems). The sequencing-PCR mix for one sample was:

30 ng PCR product (or: 0.5 µg plasmid DNA)

1.5 µl 10 pM primer

2 µl 5 x buffer (kit component)

2 µl premix (kit component) ad 10 µl ABI-H_2O

The thermal profile for the PCR reaction was: 40 cycles of denaturation at 96 °C for 30 sec, annealing at ≤ 60 °C for 15 sec, and extension at 60 °C for 4 min, followed by final extension at 60 °C for 2 min. Sequencing products were purified by ethanol precipitation (see section 4.1.3.) and analyzed in a ABI prism sequencer (Perkin Elmer).

4.2 Manipulation of RNA

4.2.1 Isolation of RNA

10 ml LB cultures were grown to mid-log phase, i.e. to an optical density at 600 nm of 0.5-0.6. 2 mL of culture was mixed with 4 mL of RNA Protect Reagent (Qiagen), vortexed, and centrifuged at room temperature, 6000 x g for 10 min to pellet cells. The supernatant was removed and cell pellets stored at -80 °C for 1 week or less before RNA extraction.

RNA isolation was carried out as directed in the QIAGEN RNeasy Mini protocol with the modifications that cell pellets were resuspended in TE buffer containing lysozyme (400 µg/ml). To remove traces of chromosomal DNA, 60 mg total RNA was treated with 55 U RNase-free DNase I (Roche Diagnostics GmbH, Mannheim, Germany) for 2 h at 37 °C. The RNA was purified using the RNeasy Mini Prep™ clean-up protocol as recommended by the manufacturer (Qiagen). The quality of the isolated RNA was analyzed by agarose gel electrophoresis and using the Bioanalyzer 2100 (Agilent Techn., Palo Alto, CA, USA). RNA concentrations were determined spectrophotometrically and all samples were stored at -80 °C. As a control for successful removal of all DNA from the samples, 2 µl of the DNase treated RNA or 1 µl DNA as positive control were used as template in a PCR reaction with primers binding within the coding sequence of gene *fimH*. The DNase digest was considered as complete if no product could be amplified from the RNA samples.

4.2.2 Northern hybridization

For Northern blotting, RNA was separated by denaturing electrophoresis. A 1.2 % agarose/formaldehyde gel was prepared by mixing 1.95 g agarose and 96 ml DEPC-treated H_2O. The agarose was dissolved by heating in a microwave, mixed well, and cooled to 60 °C. In a fume hood, 11.05 ml of 37 % formaldehyde, and 13.3 ml freshly prepared 10× MOPS buffer were added, mixed well and poured into the gel casting device.

10-20 µg DNase-treated RNA in a final volume of 10 µl were mixed with an equal volume of 2× RNA loading buffer and denatured at 55 °C for 15 min. The RNA samples were then loaded onto the formaldehyde gel and separated in 1 x MOPS running buffer at 4 V/cm electrode distance for 4-5 hours, until the bromophenol blue dye had migrated three-quarters of the way down the gel. After electrophoresis was completed, the gel was stained with ethidium bromide and photographed to record the electrophoretic separation of the loaded RNA.

RNA was transferred to a nylon membrane by overnight blotting as described earlier for Southern hybridization (see section 4.1.14.), with minor modifications: RNA transfer does not need depurinization, and the composition of the denaturation- and neutralization buffers differed slightly. The nylon membrane was prepared for hybridization as described for Southern hybridization analysis.

DNA probes were amplified by PCR, followed by ECL-labeling, and hybridization to the membrane over night at 42 °C. After washing with 0.4× SSC/0.1 % SDS, transcripts were detected using the ECL advance nucleic acid detection kit (Amersham Biosciences) following the manufacturer’s recommendations.

4.2.3 Determination of the transcription starting point.

To determine the transcription starting point of *matA*, the 5'-RACE kit from Roche Applied Science (Cat. No. 03 353 621 001, Germany) was used. This method is used to extend partial cDNA clones by amplifying the 5' sequences of the corresponding mRNAs. During PCR, the thermostable DNA polymerase is directed to the appropriate target RNA by a single primer derived from the region of known sequence; the second primer required for PCR is complementary to a general feature of the target - in the case of 5'-RACE, to a homopolymeric tail added (via terminal transferase) to the 3'-termini of cDNAs transcribed from a preparation of mRNA. This synthetic tail provides a primer-binding site upstream of the unknown 5'-sequence of the target mRNA. The products of the amplification reaction are cloned into a plasmid vector for sequencing and subsequent manipulation.
We followed the manufacturer's protocol, with slight modifications. 2 µg of RNA were used in each experiment. The primers used are in the Table 8.

Table 8: Primers used for the 5-RACE method.

Name	Sequence (5'-3')
SP1	CGCCGATGGGTATACACTG
SP2	CCGCAATCAATACGACCTG
SP3	GTAGGCGTCATGGGGAGACC
SP4	CGGAAGTAAATAAGATACG

The Dap polymerase (Roche) was used for the nested PCR.
Prior to subcloning, the PCR products were cut out from an agarose gel and purified on a column (Quiagen PCR purification kit).
The primer SP4 was used to get only the second transcriptional start point product of strain IHE3034 by PCR.

4.2.4 cDNA synthesis and hybridizations

For cDNA synthesis, the Superscript III reverse transcription kit (Invitrogen) was used. RNA was transcribed into cDNA and concomitantly fluorescence labeled by the

incorporation of Cy3 and Cy5 (GE Healthcare, Amersham Biosciences, Freiburg, Germany).
10 µg of total RNA in a final volume of 15 µl were mixed with 1 µg of random hexamer primers (Amersham Biosciences). This annealing mix is heated for 10 minutes at 70 °C, then cooled down to room temperature for 5 min.
The composition of the RT-mix for 1 sample was:

Reaction mix:
5x first strand buffer* 8 µl
0.1x DTT* 4 µl
RNaseOut 1 µl
Nucleotide mastermix 4 µl
Cy3- or Cy5-dUTP (1 mM) 4 µl
SuperScript III TM (200 U/µl) 2 µl
Annealing mix 15 µl

Total volume 38 µl

* first strand buffer and DTT is included with purchase of Superscript III

The reaction mix is incubated at 50 °C for 30 min. After a brief centrifugation, 2 µl of SuperScript III TM is added again and the mix is incubated for additional 30 min at 50 °C. The reaction is stopped by incubated 15 min at 70 °C. To hydrolyze the RNA, 10 µl NaOH (1 M) is added and the mix incubated at 65 °C for 15 min. Afterwards the reaction is cooled down to room temperature. To neutralize, 25 µl Tris-HCl (1M, pH 7.5) has to be added.

Uncoupled dye was removed by another purfication using the PCR cleanup kit. Concentration of cDNA and amount of incorporated dye was measured for each sample using a Nanodrop spectrophotometer (Ambion).

4.2.5 DNA microarray hybridization and analysis

Transcription profiling was performed using the *sciTRACER E. coli* K-12-patho chip (Scienion AG, Berlin, Germany) which comprises 3340 PCR products derived from 3840 genes from the database of *E. coli* K-12 strain MG1655 (acc-nr: NC_000913). The PCR products designed by the Scienion bioinformatics department cover 87 % of the realized ORFs. Additionally, 370 PCR products from various genomes of pathogenic *E. coli* are also present on the chip. All PCR products were dissolved in spotting solution and spotted robotically onto modified glass slides. All fragments were purified, normalized and they are represented in single spots on each array. Multiple controls were spotted in the last row of each subarray block forming the control area and comprised controls enabling spiking and endogenous controls.
The *sciTRACER E. coli* K-12-patho chip represents a custom tailored microarray and contains oligonucleotide probes that cover the complete genome of *E. coli* K-12 strain MG1655 and several genomes of *E. coli* variants pathogenic for humans and birds.

Microarrays were hybridized with the fluorescence-labeled probes for 2 days at 49 °C in total darkness, and afterwards washed according to the manufacturer's protocol. The microarrays were scanned using the GenePix4000B microarray reader (GE Healthcare). Data were linearly normalized with EMMA2.0 software (78). Genes were considered to be up-regulated when RNA level was found to be increased at least twofold in the wild type in comparison to the mutant. Genes were regarded to be negatively affected when the RNA level was at least twofold higher. Data analysis was repeated on at least 4 independent microarray experiments for each condition tested, with dye-switch. The normalization was done under the function LOWESS. The statistical significance or p-value for the genes for the data was $p < 0.05$, using a t-test with the Holm function (the Bonferroni function gave appreciatively the same results). In total, 11 independent microarrays were evaluated and the mean for each single gene was calculated. The Hierarchical Clustering is done using the EMMA2.0 software too (defaults: mean, euclidian, p=0.01).

In case of the Operon chips used for the transcriptome analysis between IHE3034 and its isogenic *matA* mutant, the protocol furnished by the manufacturer was followed, with slide modification. The labelling was performed like previously explained, with a quantification of the dye integration and normalization between each probes. The hybridization was done at 42 °C and for 20H00. Four independent experiments were performed. In this case, data were linearly normalized with Acuity 4.0 software. Genes were considered to be up-regulated when RNA level was found to be increased at least twofold in the wild type in comparison to the mutant. Genes were regarded to be negatively affected when the RNA level was at least twofold higher. Data analysis was repeated on 4 independent microarray experiments for each condition tested, with dye-switch. The normalization was done under the function LOWESS. The statistical significance or p-value for the genes for the data was $p < 0.05$, using a t-test with the Holm function (the Bonferroni function gave appreciatively the same results).

4.2.6 Real-time RT-PCR

To confirm the results of the microarray analysis, the relative expression levels of genes were validated by real-time PCR. In brief, the DNase-digested RNA was transformed into cDNA using the iScript cDNA synthesis kit (Biorad) following the manufacturer's protocol. The transcription profiling was carried out using the iCycler iQ real-time PCR detection system (Bio-Rad Laboratories GmbH, München, Germany), according to the manufacturer's instructions. Appropriate dilution series for each of the primers were made and one tenth of the synthesized cDNA together with the iQ supermix (Bio-Rad) for the real-time PCR. The nucleotide sequences of employed primers are listed in Table.

Table 9: Primers for real-time RT- PCR

Name	Sequence (5'-3')
flhD left rt	CTCCGAGTTGCTGAAACACA
flhD right rt	GTGGCTGTCAAAACGGAAGT
cadA left rt	GAATTCCAGCACGCTACCAT
cadA right rt	ACCGCTCATACCGCATTTAC
matA left rt	GAGTACAGCTTGGCCTCTGC
matA right rt	CGCTGGACTGAGTCGTGATA
matB left rt	CCGCTGATGATGGAGAAAGT
matB right rt	CGCCTTATCACCAACACCTT

Table 9: Primers for real-time RT- PCR – continued

matC left rt	TCAAACCAGACAATGCGGTA
matC right rt	CGCCTCATCAATATCCGTCT
matD left rt	TGTCGAGTTTGTTCGCTGAC
matD right rt	CAGCAAATAGGTGGGGTGAT
matE left rt	CCGAGGTTCACCATCTGTTT
matE right rt	CTCAGCAACACCTCAATCCA
matF left rt	TAGCACTGATGGCAATACGG
matF right rt	AAGTCAGCGCTTCAGGAGAG
fliC RT left	TTGATGAAATTGACCGCGTA
fliC RT right	CGTTGCAGCTTTGTTGGTAA
fliA RT left	AGCGTGGAACTTGACGATC
fliA RT right	CTATTGCCTGTGCCACTTCA
cheZ RT left	CGCAGGATTTTCAGGATCT
cheZ RT right	CCTGATCCTGACTGGCTACC
cheY RT left	GCGTAACCTGCTGAAAGAGC
cheY RT right	CGCTTCTGCAGTCACCATTA
frr left for real time pcr	GGCAAGCGTAACGGTAGAAG
frr right for real time pcr	CTTGTTCTGCTTCACCACGA
cysW left	ACTGCTGACGCTACTGGACA
cysW right	GACACGTCACGAAGATGGTG
cysK left	AAGCTGCTGAAAGCGTTAGG
cysK right	CAACCTGACCGTCGGTATCT
aer left	AGAAAATGTTGCCCATCAGG
aer right	AGCGTCTCACTGCCATTTCT
tar left	CGGGTGAAGTGCGTAATCTT
tar right	GATGCAATCTCGCCCATAAT
pyrI left	TTGGTCTGAACCTGCCTTCT
pyrI right	CAGACCAGCACATTGTCGAT
hdeA left	TGCTTCTTCTGCCAGTTGTG
hdeA right	ACGGTTGCAATACCCTGAAC
hdeB left	GCCAATGAATCCGCTAAAGA
hdeB right	CAAATTTTTCTGCGGGTTTT
glnB left	ACTGGCCGAAGTCGGTATTA
gnlB right	GCCGTGCGAATAATGGTATC
hdhA left	ATTTTCGCCGTGCTTATGAA
hdhA right	CCAGGTCAAACGCCATATTT
motA left	TTTCCTCGGCATTTTATTGG
motA right	GCACATGCTCTTCCAGTTCA
yedU left	TGCAGCAATCTTTGTTCCTG
yedU right	CCAATCTCTGGCGTTTGTTT

4.2.7 Semi-quantitative reverse-transcription PCR (RT-PCR)

cDNA samples derived from reverse transcription were 20× diluted in water and directly used for PCR amplification. As a control for DNA contaminations, a second PCR reaction was performed using total RNA without any reverse transcription reaction. DNA served as positive control for the PCR reaction. For adjustment of cDNA amounts, 16 S

rRNA (*rrsA*) was used as internal standard using 35 cycles of amplification. The RT-PCR primers were selected with the FastPCR software version 3.6.28 (Ruslan Kalendar, Institute for Biotechnology, University of Helsinki, Finland). The sequences of all oligonucleotides used are listed in Table 10.

Table10: Primer use for the RT-PCR experiments.

Name	Sequence (5'-3')
matA-B left	CCTCGATAGCCACGTCAAAT
matA-B right	CGCTGGACTGAGTCGTGATA
matA-C left (use in pair with matA-B right)	GCGTCGCATTTCCTGTATTT
matDE left	GCTTTCGTATGGGTGACGTT
matDE right	TTACCGTCTCCGGTCGTATC
matEF left	CACCTGATCGAAGGACCATT
matEF right	AACGTCACCCATACGAAAGC
matAD left	ACCAATCCCGTACAGTGAGC
matAD right	CGCTGGACTGAGTCGTGATA

For the PCR reaction, Red *Taq* polymerase ready mix (Sigma) was used (see section 4.1.5.). The 2× concentrated mix contained all necessary components for PCR, thereby minimizing pipetting errors. 10 µl of the ready mix were mixed with 4.6 µl water and 0.2 µl of each 100 pM RT primer. This mixture was then added to the adjusted cDNA in a final volume of 5 µl and placed into the thermal cycler.

Thermal profile: Initial denaturation 2 min, 95 °C

1. Denaturation 30 sec, 95 °C
2. Annealing 30 sec, 57-59 °C
3. Elongation 30 sec, 72 °C

(steps 1–3: 25-35 cycles)

Final elongation 2 min, 72 °C

Since the PCR ready mix already contained a loading dye for gelelectrophoresis, 10 µl of the PCR samples were directly loaded on a 2 % agarose gel and analyzed after ethidium bromide staining. To keep the PCR amplification in a linear range, i.e. to prevent over-saturation of the PCR products, the cycle number of amplification was altered, whereas

the template amount was always kept constant. The primers used are listed in the table 10.

4.3. Working with Protein

4.3.1 Denaturing polyacrylamide gel electrophoresis (PAGE)

Protein samples were separated and analyzed in denaturing polyacrylamide gels as described by Laemmli (1970). This was done using the detergent sodium dodecylsulfate (SDS), which binds to proteins and leaves them unfolded and negatively charged. Therefore, the protein mixture can be separated in the meshwork of the polyacrylamide gel due to variable migration speed depending on the size of the protein. Since most of the proteins of interest in this study were about 15 kDa in size, a 15 % resolution polyacrylamide gel was used. The size of the gels was 10 × 10 × 0.5 cm, and electrophoresis was performed at room temperature at 16 mA per gel in 1x electrophoresis buffer. The samples were loaded onto the gel after heating for 10 min at 90 °C in 1× loading buffer.
The acrylamide gel consists of two parts: a lower part mediating the separation of the proteins, and an upper part, which is used for concentration of the sample in a single running front after entering the gel. The mixture sufficient for 4 mini gels was:

15 % separation gel:	15 ml 30 % acrylamyde: bis-acrylamide (37.5:1)
	5 ml 1.5 M Tris-HCl, pH 8.8
	10 ml dH_2O
	300 µl 10 % (w/v) SDS
	250 µl 10 % (w/v) ammonium persulfate (APS)
	8 µl TEMED
5 % collecting gel:	1.96 ml 30 % acrylamyde: bis-acrylamide (37.5:1)
	2.8 ml 0.5 M Tris-HCl, pH 6.8
	4.6 ml dH_2O

	112 µl 10 % (w/v) SDS
	32 µl 10 % (w/v) APS
	16 µl TEMED
10 x running buffer:	30 g Tris
	144 g glycine
	10 g solid SDS
	ad 1 l dH_2O
4 x SDS-loading buffer:	2.5 ml 1 M Tris-HCl, pH 6.8
	4 ml 50 % (v/v) glycerol
	0.8 g solid SDS
	0.1 ml ß-mercaptoethanol
	0.02 g bromophenol blue
	ad 10 ml dH_2O

4.3.2 Visualization of proteins in acrylamide gels by Coomassie staining

After electrophoresis, the polyacrylamide gels were incubated for 15 min in Coomassie staining solution. Protein bands were visualized after removing unbound Coomassie dye by incubating the gel in destaining solution I for 30 min, followed by incubation in destaining solution II for > 2h.

Staining solution:	1 g Coomassie Brilliant Blue R-250
	0.25 g Coomassie Brilliant Blue G-250
	238 ml ethanol
	50 ml acetic acid
	ad 500 ml dH_2O
Destaining solution I:	10 % (v/v) acetic acid; 50 % (v/v) ethanol
Destaining solution II:	7.5 % (v/v) acetic acid; 5 % (v/v) ethanol

4.3.3 Immunoblotting

For the preparation of crude cell extracts, 800 µl bacterial culture were centrifuged for 2 min at 13,000 rpm in a table top centrifuge and the pellet was resuspended in ¼ vol of the measured optical density of the culture in 1× Laemmli buffer (e.g. 250 µl for a sample with OD_{600nm} = 1). After heating the samples for 10 min at 90 °C, 15 µl were used for PAGE (see section 4.3.1.). After PAGE, separated proteins were transferred to a nitrocellulose membrane (Optitran BA-S85 Reinforced NC; pore size 0.45 µm; Schleicher&Schuell). The transfer of the proteins was carried out between two graphite plates in a semi-dry western blotting apparatus, using 12 Whatman paper slices, soaked with Anode buffer I, II or Cathode buffer. The membrane was incubated for 5 min in Anode buffer II. The lower graphite plate (anode) was moistened with water and covered with 6 slices of Whatman paper soaked with Anode buffer I, followed by 3 slices of Whatman papers soaked with Anode buffer II. The nitrocellulose membrane was laid on top of the Whatman papers, followed by the polyacrylamide gel and 3 slices of Whatman paper soaked in Cathode buffer. Air bubbles were carefully removed before laying the second graphite plate at the top (cathode). The transfer was carried out by applying an electric current of 0.8 mA cm^{-2} for 1 h.

Anode buffer I: 0.3 M Tris, 20 % methanol
Anode buffer II: 25 mM Tris, 20 % methanol
Cathode buffer: 25 mM Tris, 40 mM ε-amino-n-capronic acid, 20 % methanol

After transfer of the proteins, the membrane was incubated over night at 4 °C (or 1 h at room temperature) in TBS-T solution (0.05 M Tris-HCl, pH 7.5; 0.15 M NaCl; 0.1 % Tween 20) supplemented with 5 % fat-free dry milk. Subsequently, the blot was incubated with the primary antibody for 1 h at room temperature. The concentration of the primary antibody depended on the titre, but the dilution usually ranged from 1:1000 to 1:5000 in TBS-T supplemented with 5 % dry milk. After washing the membrane with TBS-T three times for 5 min, the secondary peroxidase-conjugated antibody (1:5000 diluted in TBS-T) was added and incubated for 1 h at room temperature. Finally, the membrane was washed three times for 10 min with TBS-T at room temperature. Signal

detection using the ECL kit (Amersham Biosciences) was performed as described before (see section 4.1.14.).

4.4. Phenotypic assays

4.4.1 Detection of aerobactin expression

For aerobactin media plates, 1 ml of an overnight culture (YT, Tc) of the indicator *E. coli* strain EN99 was mixed with 100 ml aerobactin soft agar (0.75 %(w/v), see section 3.3.1) medium, pre-cooled to 42 °C. A thin layer (0.5 mm) of the mixture was poured on the surface of the aerobactin plates. The bacterial strains to be tested were grown overnight in 1 ml LB medium. Sterile susceptibility discs (Oxoid) were soaked with cells of the overnight culture and were placed on the aerobactin plates and incubated overnight at 37 °C. Aerobactin production was assessed by the presence of growth zones of the iron-deficient indicator strain EN99 around the colonies of the tested strains.

4.4.2 Detection of type 1 fimbriae expression

Overnight cultures of the strains to be tested and of a positive (*E. coli* strain Nissle 1917) and of a negative (*E. coli* strain AAEC189) control were grown. The mannose-dependent yeast agglutination assay was carried out by mixing 10 µl of the different bacterial overnight cultures with 10 µl yeast cells-suspension (1 mg/ml *Saccharomyces cerevisiae* cells diluted in 0.9 % (w/v) NaCl, with or without 2 % (w/v) mannose) on microscope slides (75:25:1 mm). The slides were kept on ice until the aggregation of bacterial and yeast cells was observed in absence of mannose.

4.4.3 Detection of F1C fimbriae expression

Overnight cultures of the strains to be tested and of a positive (*E. coli* strain Nissle 1917) and of a negative (*E. coli* strain AAEC189) control were grown. For the

immunoagglutination assay a polyclonal α-F1C fimbriae rabbit antibody was used (provided by S. Kahn, Wuerzburg), that was diluted 1:1000 in 1× PBS. The immunoagglutination assay was carried out by mixing 10 µl of the bacterial overnight culture with 10 µl of the α-F1C fimbriae antibody solution on microscope slides (75:25:1 mm) and incubation on ice until the aggregation of the bacterial cells was clearly observed.

1× PBS
NaCl 8g
KCl 0.2 g
Na_2HPO_4 1.4 g
K_2HPO_4 0.24 g
NaOH add to pH 7.4
dH_2O to a final volume of 1000 ml

4.4.4 Detection of S fimbriae expression

Overnight cultures of the strains to be tested and of a positive (*E. coli* strain Nissle 1917) and of a negative (*E. coli* strain AAEC189) control were grown. For the immunoagglutination assay a polyclonal α-S fimbriae rabbit antibody was used (provided by S. Kahn, Wuerzburg), that was diluted 1:1000 in 1× PBS. The immunoagglutination assay was carried out by mixing 10 µl of the bacterial overnight culture with 10 µl of the α-F1C fimbriae antibody solution on microscope slides (75:25:1mm) and incubation on ice until the aggregation of the bacterial cells was clearly observed.

4.4.5 Biofilm formation experiments

Bacterial strains were grown overnight at 37 °C in LB. Overnight cultures were diluted (1:200) with fresh LB or M63 medium. 160µl of the 1: 200 bacterial dilution was added in wells of a 96-well microtiter plate (Flexible Plate, U-Bottom, Lid for flexible plate, Falcon). Sterilization of the 96-well microtiter plate and lid by exposure to UV light for 20 min before use. The cells were grown at 20 °C or 37 °C without agitation. After 24H (37 °C) or 48H (20 °C), the supernatant of the biofilm was completely discarded and the

biofilm washed twice with 1× PBS. Finally, the biofilm was dried at 60 °C, each well stained with 160µl of the 0.1% Kristal Violet solution (in water) for 10 min and washed at least 2 times with 1× PBS. The absorbed Kristal Violet is eluted with 180µl of acetone/EtOH (1/5 v/v). The biofilm is measured at OD_{580}.

4.5 *In silico* analysis

For standard sequence comparison and similarity searches, the Basic Local Alignment SearchTool (BLAST) at the National Center for Biotechnology Information Homepage was used.
For alignments of nucleotide and amino acid sequences, the BioEdit sequence alignment editor V7.0.1 was used (129). Genome comparison was performed using the Artemis Comparison Tool (ACT) Release 4 of the Sanger Institute.

4.6 Statistical analyse

Statistical analyses were performed using the Prism 4.0b software package (GraphPad Software). The statistical significance or p-value for the data was $p < 0.05$.

5. Results

5.1 Analysis of zoonotic risk between human and avian ExPEC

ExPEC may cause different diseases in humans and animals thus bearing a zoonotic risk. Assuming that host specificity is due to (i) specific genes required for metabolism or pathogenicity, (ii) allelic variation of genes coding for proteins interacting with the host and (iii) the ability to sense the host and specifically regulate virulence gene expression, our aim was to find out whether there is a host specificity of human and avian ExPEC strains.

As specific virulence factors that trigger the infection of either humans or poultry could not be identified yet, we investigated whether differential regulation of virulence gene expression may be responsible for host specificity of APEC and human ExPEC strains. A thorough analysis of factors that contribute to the host specificity (human vs. poultry) of ExPEC strains was therefore important to evaluate the zoonotic risk emerging from these bacteria. In this context, the difference between the human and the avian body temperature (37°C and 41-42°C, respectively) can be important. One focus of this study was therefore the transcriptome analysis of a human and an avian ExPEC isolate of serotype O18:K1 in response to different growth temperatures by means of DNA arrays.

The aim of these experiments was the identification of genes that are specifically transcribed either in response to the body temperature of humans (37 °C) and avians (41-42 °C) or depending on the strain background. Subsequently, differential regulations of promising candidate genes identified by the global transcriptome analysis were then confirmed by additional approaches.

5.1.1 The characterisation of human and avian ExPEC strains

The COLIRISK project members characterised nine human and avian ExPEC strains that have been compiled for the COLIRISK strain collection. In Table 11 the preliminary results performed by the COLISK partners for this approach are summarised. According to these results, a clear correlation between the presence of ExPEC-specific virulence-associated genes and human or avian origin could not be identified.

Table 11: Genotypic characterisation of the human and avian ExPEC isolates of the COLIRISK strain collection.

	RS218 Human O18:K1	**IHE3034** Human O18:K1	**BEN79** Avian O18:K1:H7	**BEN374** Avian O18:K1:H7	**IHE3072** Human O2:K1:H5	**BEN2908** Avian O2:K1:H7	**1772** Avian O2:K1	**285** Human O78	**789** (AC/I) Avian O78
hly	+	-	-	-	-	-	-	-	-
cnf1	+	-	+	-	-	-	-	-	+
cdt	-	+	+	+	-	-	-	-	-
pap	+	-	-	-	+	-	-	-	-
sfa	+	*sfaII*	+	+	+	-	-	-	*fac*
fim	+	+	+	+	+	+	+	+	+
ybn	+	+	+	+	+	+	+	+	+
iro	+	+	+	+	+	+	+	+	+
iut	+	+	+	+	+	+	+	+	+
cvaC	-	-	+	+	+	+	-	+	-
traT	+	+	+	+	+	+	-	-	-
kps	K1	K1	K1	K1	K1	II	K1	II	-
ibeA	+	+	+	+	-	+	+	-	-
flg	+	+	+	+	+	+	+	+	+
fimH	+	+	+	+	+	+	+	+	+
matB	+	+	+	+	+	+	+	+	+
csgA	+	+	+	+	+	+	+	+	+
csgD	+	+	+	+	+	+	+	+	+
sfaH	+	+	+	+	-	-	-	-	+
sfaS	+	+	+	+	-	-	-	-	-
focG	+	+	+	+	-	-	-	-	-
focH	+	+	+	+	-	-	-	-	+
papGII	-	-	-	-	+	-	-	-	-
papGIII	+	-	-	-	-	-	-	-	-

The presence or absence of different fimbrial adhesin genes was investigated by PCR assays (Table 11). All strains tested carried the determinants coding for type I fimbriae (*fim*) as well as for curli (*csg*) and Mat fimbriae (*mat*). The O2:K1 strains were

negative for both, the S- and F1C-fimbrial gene cluster. Strain 789 was PCR-positive for the adhesin genes *foc*H and *sfa*H but negative for the accessory genes *sfaS* and *focG*. This is due to the fact that this strain carries the highly homologous *fac* gene cluster which is also a member of the S adhesin family.

Furthermore, phenotypic tests have been performed to test the COLIRISK strains for production of α-hemolysin, the iron-uptake system aerobactin, type 1- and F1C fimbriae, and for their motility (Table 12).

Table 12: Phenotypic characterisation of the COLIRISK strains.

Strain	Origin	Serotype	*Type I fimbriae*	*F1C fimbriae*	*S fimbriae*	*P fimbriae*	*Colicins*	*flg*
RS218	hu	O18:K1	+	+	+	+	-	+
IHE3034	hu	O18:K1	+	+	*sfa*II +	+	-	+
BEN79	av	O18:K1:H7	+	+	-	+	+	+
BEN374	av	O18:K1:H7	+	+	-	+	+	+
IHE3072	hu	O2:K1:H5	+	+	-	+	+	+
BEN2908	av	O2:K1:H7	+	+	-	+	+	+
1772	av	O2:K1	-	-	-	+	-	+
285	hu	O78	+	+	-	+	+	-
789 (AC/I)	av	O78	-	-	*fac*	+	-	-

+: expressed; -: not expressed. K1: serotype of capsule. II: Group II capsular polysaccharides.

To complete this analysis, the expression of these fimbriae (when the genes were present) was compared in the strains isolated from humans or poultry. Polyclonal antibodies specific for these fimbriae were used to perform agglutination assays. Furthermore, the expression of other virulence-associated traits such as the siderophore aerobactin, curli fimbriae, and cellulose formation was tested in order to see differences in gene expression between the different strains (Table 12). For this, all the strains were inoculated on specific agar plates (CAS plate, Congo Red plate, aerobactin plates) and incubated at different temperatures, including the human and avian body temperature, but also 20 °C and 30 °C.

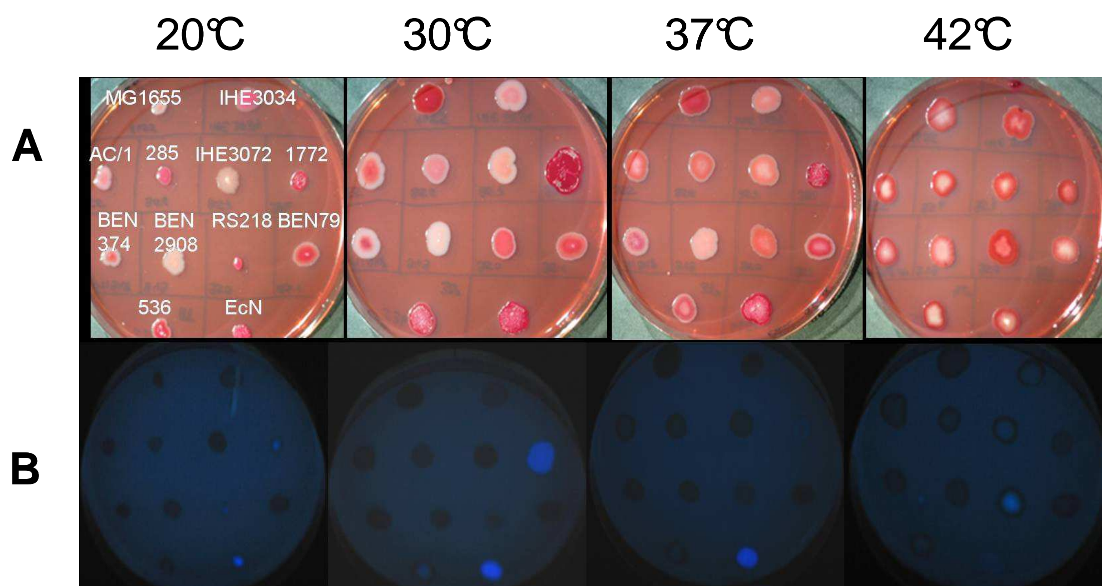


Figure 13: Analysis of rdar morphotype and cellulose expression. Figure A depicts the rdar (red dry and rough) morphotype, i.e. the simultaneous expression of curli and cellulose on Congo Red agar plates after 48 h of growth). Figure B indicates cellulose expression on calcofluor agar plates after 48 h of growth. The *E. coli* strain EcN expresses at 30 °C and 37 °C the so-called rdar morphotype on congo red agar plates (370) which is characterized by red colored colonies with a dry colony surface and strong cell-cell interactions. This morphotype is considered to be due to co-expression of curli fimbriae and cellulose. In comparison, the uropathogenic *E. coli* strain 536 expresses the rdar morphotype only at 30 °C. The *E. coli* K-12 MG1655 is our negative control for cellulose and expresses curli at 30 °C.

The results (see Figure 13) suggested there was no clear correlation between the strain origin and rdar morphotype expression. Cellulose biosynthesis is known to play a significant role in biofilm formation, but does not contribute to the virulence of *Salmonella enteritidis* (307). Curli fimbriae are predominantly expressed in human and avian isolates between 20 °C and 37 °C. Cellulose expression was not frequently observed, and its expression was higher at 30 °C than at lower or higher temperatures.

5.1.2 Detection of allelic variation of genes of the ExPEC patho-gene pool.

Host specificity may not only result from the presence or absence of certain virulence-associated genes or their strain-specific regulation. Differences in receptor recognition due to amino acid variation within the adhesin molecule may also contribute

to variation of the receptor binding ability (allelic variation, pathoadaptive mutations). The following fimbrial determinants (genes) of ExPEC strains were studied with regard to allelic variation: type 1 fimbriae (FimH), P fimbriae (Pap/PrfG), S adhesin family (SfaS/H and FocG/H), curli fimbriae (CsgA/D) and the Mat fimbriae (MatA/B). The amino acid sequences of these fimbrial subunits have been deduced from the relevant nucleotide sequences that were determined for the members of the COLIRISK strain collection. The amino acid sequences obtained were compared to the corresponding sequences of *E. coli* K-12 strain MG1655. For FimH, the adhesin of type 1-fimbriae, sequence variations have been observed at positions V27→A, S62→A, G66→C, N70→S, S78→N and G159→S. These amino acids changes have been identified to confer shifts in receptor specificity (295) and/or virulence (145). The sequence variation at position 62 is especially important for adhesiveness of meningitis-associated *E. coli* to collagen (261) and for binding of mono-mannose residues thus playing a role for extraintestinal infections. This amino acid exchange was only detected in the two human O18:K1 isolates, but not in any other human or avian isolate of the COLIRISK strain collection (see Figure 14). It can be speculated that the S62→A exchange may be involved in host specificity of human O18:K1 isolates. The other amino acid exchanges detected could not be correlated with the source of isolation or the serotype of the strains.

```
                                62  66  70      78
MG1655    TQIFCHNDYPETITDYVTLQRGSAYGGVLSNFSGTVKYSGSSYPFPTTSETPRVVYNSRT
IHE3034   TQIFCHNDYPETITDYVTLQRGAAYGGVLSSFSGTVKYNGSSYPFPTTSETPRVVYNSRT
RS218     TQIFCHNDYPETITDYVTLQRGAAYGGVLSSFSGTVKYNGSSYPFPTTSETPRVVYNSRT
BEN79     TQIFCHNDYPETITDYVTLQRGSAYGCVLSSFSGTVKYNGSSYPFPTTSETPRVVYNSRT
BEN2908   TQIFCHNDYPETITDYVTLQRGSAYGGVLSSFSGTVKYNGSSYPFPTTSETPRVVYNSRT
BEN374    TQIFCHNDYPETITDYVTLQRGSAYGGVLSSFSGTVKYNGSSYPFPTTSETPRVVYNSRT
IHE3072   TQIFCHNDYPETITDYVTLQRGSAYGGVLSNFSGTVKYSGSSYPFPTTSETPRVVYNSRT
789       TQIFCHNDYPETITDYVTLQRGSAYGGVLSNFSGTVKYSGSSYPFPTTSETPRVVYNSRT
285       TQIFCHNDYPETITDYVTLQRGSAYGGVLSNFSGTVKYSGSSYPFPTTSETPRVVYNSRT
```

Figure 14: **Amino acid sequence variations of FimH among *E. coli* strains of the COLIRISK strain collection compared to *E. coli* K-12 strain MG1655.** The substitutions are boxed.

The allelic variation proteins encoded by the curli determinant is interesting, too. The level of sequence variation observed was very low in the case of the regulator protein CsgD (S109A) but higher for the curli adhesin CsgA (T61A; N110D; E112T). Further studies are required to analyse the impact of these allelic variations on the function of the

encoded proteins. However, no correlation could be found in regards of the host, serotype and certain alteration with this amount of strain checked.

```
                           21
MG1655       VLAIALVTVFTGMGVAQAADVTAQAVATWSATAKKDTTSKLVVTPLGSLAFQYAE
RS218        VLAIALVTVFTGTGVAQAADVTAQAVATWSATAKKDTTSKLVVTPLGSLAFQYAE
BEN79        VLAIALVTVFTGTGVAQAADVTAQAVATWSATAKKDTTSKLVVTPLGSLAFQYAE
BEN374       VLAIAXVTVFTGTGVAQAADVTAQAVATWSATAKKDTTRKLVVTPLGSLAFQYAE
IHE3034      VLAIALVTVFTGTGVAQAADVTAQAVATWSATAKKDTTSKLVVTPLGSLAFQYAE
IHE3072      VLAIALVTVFTGTGVAQAADVTAQAVATWSATAKKDTTSKLVVTPLGSLAFQYAE
BEN2908      VLAIALVTVFTGTGVAQAADVTAQAVATWSATAKKDTTSKLVVTPLGSLAFQYAE
789          VLAIALVTVFTGMGVAQAADVTAQAVATWSATAKKDTTSKLVVTPLGSLAFQYAE
285          VLAIALVTVFTGMGVAQAADVTAQAVATWSATAKKDTTSKLVVTPLGSLAFQYAE
1772         VLAIALVTVFTGMGVAQAADVTAQAVATWSATAKKDTTSKLVVTPLGSLAFQYAE

                           53 55                                 93
MG1655   GIKGFNSQKGLFDVAIEGDSTATAFKLTSRLITNTLTQLDTSGSTLNVGVDYNGAAVEKT
RS218    GIKGFNSQKGLFDVAIEGDSTATAFKLTSRLITNTLTQLDTSGSTLNVGVDYNGATVEKT
BEN79    GIKGFNSQKGLFDVAIEGDSTATAFKLTSRLITNTLTQLDTSGSTLNVGVDYNGATVEKT
BEN374   GIKGFNSQKGLFDVAIEGDSTATAFKLTSRLITNTLTQLDTSGSTLNVGVDYNGATVEKT
IHE3034  GIKGFNSQKGLFDVAIEGDSTATAFKLTSRLITNTLTQLDTSGSTLNVGVDYNGAAVEKT
IHE3072  GIKGFNSQKGLFDVAIEGDSTATAFKLTSRLITNTLTQLDTSGSTLNVGVDYNGAAVEKT
BEN2908  GIKGFNSQKGLFDVAIEGDSTATAFKLTSRLITNTLTQLDTSGSTLNVGVDYNGAAVEKT
789      GIKGFNSQKGLFDVATESDSTATAFKLTSRLITNTLTQLDTSGSTLNVGVDYNGAAVEKT
285      GIKGFNSQKGLFDVAIEGDSTATAFKLTSRLITNTLTQLDTSGSTLNVGVDYNGAAVEKT
1772     GIKGFNSQKGLFDVAIEGDSTATAFKLTSRLITNTLTQLDTSGSTLNVGVDYNGAAVEKT
```

Figure 15: Amino acid sequence variations of MatB among *E. coli* strains of the COLIRISK strain collection compared to *E. coli* K-12. The substitution positions are boxed (S21R; I53T; G55S; A93T. Mutation in the N-terminal signal: T-6M).

Amino acid sequence alterations (see Figure 15) have also been detected for MatB (S21→R, I53→T, G55→S, A93→T). MatB exhibits a high amount of mutations which may also contribute to variation of the receptor binding ability (allelic variation or pathoadaptive mutations). But the receptor of Mat fimbriae is not yet identified to answer this question. Nevertheless, no correlation could be found so far with regard to the host or its serotype.

5.1.3 Biofilm formation on polyethylene

Biofilm formation is, as well as invasion, considered a potential cause of recurrent and chronic infections enabling the bacteria to stay within the body and to escape the host's immune system and antibiotic therapy.

The COLIRISK strains were tested for their ability to form biofilm on inert plastic surfaces. A colorimetric quantification of biofilm formation (M63B1 medium, 37 °C) in

polyethylene microtiter plates has been carried out (Fig. 16). The strains differed in their ability to form biofilm on polyethylene. There was neither a clear correlation between human and avian *E. coli* isolates nor between certain serotypes and biofilm formation.

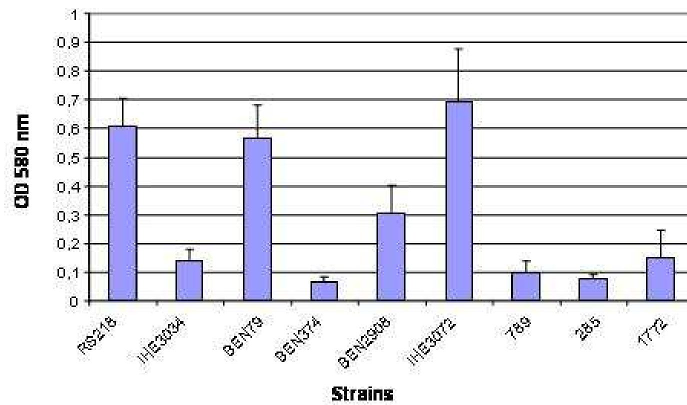


Figure 16: Colorimetric determination of biofilm formation (M63B1 medium, 37 °C) in polyethylene microtiter plates.

5.1.4 Motility tests

Furthermore, motility tests have been performed to screen the COLIRISK strains for expression of flagella and for their motility (Table 13 and Figure 17).

Table 13: Motility of the COLIRISK strains.

Strain	Origin	Serotype	Motility
RS218	hu	O18:K1	+
IHE3034	hu	O18:K1	++
BEN79	av	O18:K1:H7	++
BEN374	av	O18:K1:H7	++
IHE3072	hu	O2:K1:H5	+
BEN2908	av	O2:K1:H7	++
1772	av	O2:K1	++
285	hu	O78	-
789 (AC/I)	av	O78	-

The tests have been performed at 37 °C and 41 °C.

According to these results, a clear correlation between the motility of the different ExPEC strains and human or avian origin does not exist. It is nevertheless interesting that

motility and/or flagella expression of both O78 strains was impaired. Genome sequences of these two strains indicate the integrity and functionality of the flagella gene clusters (Eliora Ron, personal communication).

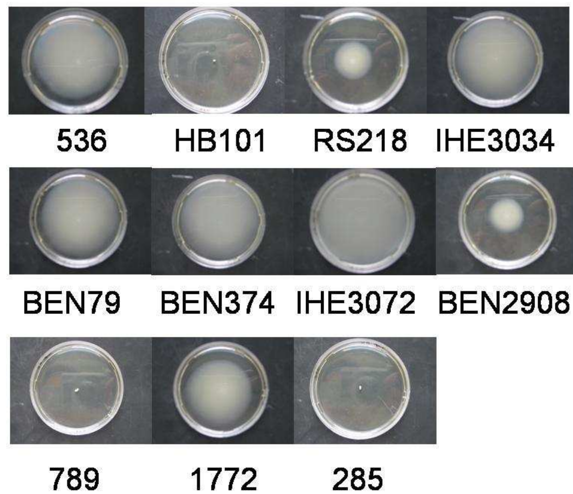


Figure 17: Motility of the COLIRISK strains. Soft agar plates (LB, 0.3% agar) were incubated at 37 °C, for 12 h. UPEC strain 536 was used as a positive control. *E. coli* strain HB101Δ*fliA* served as a negative control.

5.1.5 Transcriptome comparison of human and avian ExPEC isolates following in vitro cultivation

In order to assess whether human and avian O18:K1 isolates generally differ in gene expression and to screen for factors that may direct host specificity (infection of man or birds) the influence of growth temperature on transcription profiles of human and avian O18:K1 strains was analysed. These studies have been carried out with the strains IHE3034 (human origin) and BEN374 (avian origin) that have been grown at 37 °C (human body temperature) and 41 °C (avian body temperature). For transcriptome comparison, DNA micorarrays were used for competitive hybridisation with cy3- and cy5-labelled cDNA from total RNA extracts. These initial experiments revealed that gene expression in these strains differs at 37 °C and 41 °C (Table 14).

Table 14: Influence of human or avian body temperature on the transcriptome of *E. coli* strain IHE3034 and BEN374.

Repressed genes at 41 °C in IHE3034 and BEN374 (70 candidates genes)	Genes induced in strain IHE3034 at 41 °C (30 candidates genes)	Genes induced at 41 °C in strain BEN374 (30 candidates genes)
fli operon flagellar biosynthesis genes	*codB* cytosine transporter	*hdeB* acid resistance protein
flg operon component of flagellar motor complex	*b1498* putative sulfatase	*yedU* heat shock protein Hsp31
Chemotaxis genes (*cheA*, *cheB*, *fliZ*, *tar*, *cheZ*, *aer*, *motB*...)	*yedU* heat shock protein Hsp31	*yggB* mechanosensitive ion channel
b1409 *ynbB* putative phosphatidate cytidyltransferase	*b1171* hypothetical protein	*cdtA, B, C* cytolethal distending toxin subunits
nanA N-acetylneuraminate lyase	*hdhA* 7-α-hydroxysteroid dehydrogenase	*cadA* subunit of lysine decarboxylase
b0805 putative outer membrane receptor for iron transport	*yfeA* predicted diguanylate cyclase	*marA* multiple antibiotic resistance protein
nlp transcriptional regulator	*yggB* mechanosensitive ion channel	*cysA, D ,J, H, K, W, I, N* : sulfate and thiosulfate transport
	pyrI aspartate carbamoyltransferase	*hdeA* HdeA dimer, inactive form of acid-resistance protein, possible chaperone
	pyrL Pyr operon leader peptide	*glnB* Protein PII plays a critical role in the regulation of nitrogen metabolism
	purK N5-carboxyaminoimidazole ribonucleotide synthase	
	b1497 putative enzyme	
	napD undefined role in the post-translational assembly of a functiona NapAl	
	ORF55 Putative transposase	

Genes with significantly induced transcript levels at 41 °C relative to 37 °C were determined by competitive DNA array hybridization. The genes presented in Table 14 have an expression ratio ≥2 and a p-value ≤ 0.05. The data are representative of a least 4 experiments for each strain with dye-switch.

The hierarchical cluster analysis of the global transcription profiles of human O18:K1 isolate IHE3034 and avian O18:K1 strain BEN374 indicated that the two strains exhibit only a few differences in their transcription profiles (figure 18). The results of the transcriptome analysis in response to avian and human body temperature (41 °C vs. 37 °C) revealed a common gene set which was repressed at 41 °C including the flagellar and chemotaxis operon in both strains. The majority of genes induced at the higher

temperature differ between the human and avian isolates. Their contribution to host specificity needs to be further analysed.

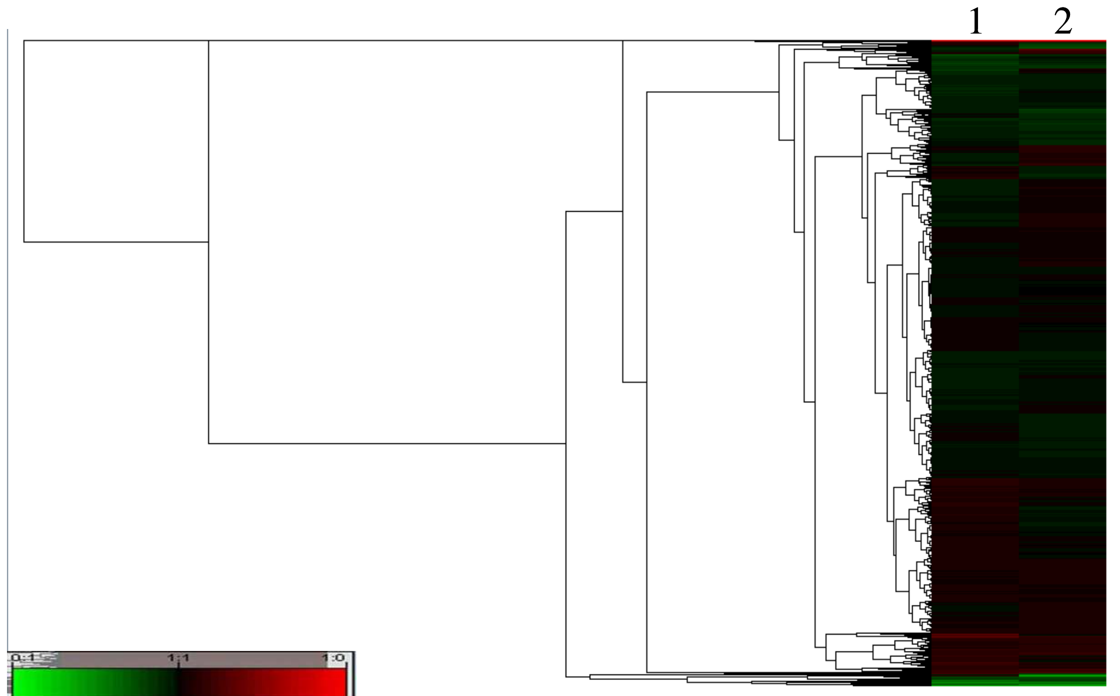


Figure 18: Cluster analysis of the transcriptome comparison of *E. coli* strains IHE3034 and BEN374 at 37 °C vs. 41 °C. The column 1 represent the avian strain BEN374 and the column 2 represent the human strain IHE3034. The hierarchical clustering was performed with the EMMA2.0 software, (defaults: mean, euclidian, p=0.01).

Some genes were specifically up or down regulated in the two strains. About the specific up regulated genes in BEN374 (see Table 15), a majority of the encoded protein are involved in metabolism (*katE*, *betB*, *deoA*, *deoC*, *galT*). The genes *betI* and *araC* code for regulatory proteins. Genes coding for conserved proteins with unknown function were present, too (*b0833* and *ybiI*). Transcript levels of genes coding for the acid resistance protein *msyB* (332) and the starvation protein *slp* involved in acid resistance (331) were upregulated, too.

Table 15: Most up-regulated genes in APEC strain BEN374 at 41 °C versus 37 °C (p≤ 0.05)

Probe designation	SignificanceTest1535 :P Dataset p value	M1 mean (log2)	M1 sd
ykgE	0.00543993	1.26974924	0.35086892
araC	0.0047973	1.54955991	0.40986838
betI	6.58E-05	1.25933291	0.07818977
cysJ	0.00888352	2.59508298	0.85196114
cysH	0.04874303	1.71133578	1.06435617
hdeB	0.00432877	1.04748358	0.26736674
cysD	0.00638976	3.68538456	1.07727588
cysA	0.01873467	2.18591424	0.93977641
ykgF	0.00752802	1.07172367	0.33184566
katE	0.00050232	1.02019865	0.12517021
cysK	0.00885973	2.15558396	0.70700166
cysW	0.02311901	1.37360156	0.63866395
cysI	0.01922881	2.87463572	1.2478343
cysN	0.02931913	1.84116217	0.93684437
ybiI	0.02354102	1.17887444	0.55186429
slp	0.00089129	1.16061294	0.17275505
msyB	0.00078219	1.08581853	0.15465298
deoA	0.00096356	1.28919875	0.19701739
cdtB	0.00034247	1.30049663	0.14029228
ME_EO_28C_*orf8*_1	0.00034578	1.23028144	0.13314663
ME_EO_28C_*orf7*_1	0.00071355	1.21381028	0.16760954
cdtC	0.00048255	1.27121131	0.15387641
deoC	0.00061479	1.32109817	0.17349344
cdtA	0.00141583	1.18962525	0.20710662
ME_EO_28C_*orf9*_1	0.00059416	1.15523527	0.14997762
betB	0.00036858	1.25641374	0.13892192
galT	0.00185744	1.19844404	0.22881511
b0833	0.00212187	1.01613398	0.20301395

sd, standard deviation

Around twelve genes were specifically down-regulated in *E. coli* BEN374 at 41 °C (see Table 16). This group of genes codes for conserved and predicted proteins (YhcH, b1773, b1760, b1742). Another gene, *map*, was strongly down-regulated in BEN374. The *map* gene encoding a methionine aminopeptidase is essential for growth in *E. coli* (55). The *csgC* was down regulated, but in contrast to the curli genes *csgAB*, it has its own promoter (EcoCyc; http://biocyc.org/) and its inactivation doesn't affect curli production (130). The other repressed genes (*ydbU*, *malE*, *yhcI*, *ycgC*) code for proteins involved in different metabolism pathways.

Table 16: Most down-regulated genes in APEC strain BEN374 at 41 °C versus 37 °C ($p \leq 0.05$)

Probe designation	SignificanceTest1535 :P Dataset: p value	M1 mean (log2)	M1 sd
map	0.00016849	-1.01514352	0.08633757
ydbU	0.00083735	-1.33476107	0.19453245
tap	0.00166095	-1.19696049	0.21999943
yhcI	0.04411655	-1.75815417	1.05019683
yhcH	0.03507661	-1.69380326	0.92384214
b1773	0.00511914	-1.19631243	0.32365422
malE	0.01985466	-1.33125959	0.58478154
cheW	0.00022486	-1.68610959	0.15795702
b1742	0.00270855	-1.13293905	0.24605796
cheR	0.00080308	-1.2037265	0.17297841
ycgC	0.00849791	-1.04375095	0.33731571
b1760	0.00107619	-1.15213419	0.18277749

sd, standard deviation

IHE3034 possesses specific genes which are up or down regulated. Only a few genes were specifically up-regulated in Human ExPEC strain IHE3034 at 41 °C (see Table 17).

Table 17: Most up-regulated genes of human ExPEC strain IHE3034 at 41 °C versus 37 °C ($p \leq 0.05$)

Probe designation	SignificanceTest1535 :P Dataset: p value	M1 mean	M1 sd
codB	0.01173818	1.40769138	0.71622353
b1498	0.00608196	1.55551794	0.65611535
b1171	0.00402307	1.20381341	0.45301183
yfeA	0.00065681	1.1317387	0.26347573
yggB	0.00097419	1.11584155	0.28780917
pyrI	0.01987001	1.46992707	0.87545328
pyrL	0.04121575	1.48148788	1.11604763
purK	0.03273331	1.09608159	0.76463036
b1497	0.0034056	1.27501449	0.45852511
napD	0.00089112	1.18312757	0.29815342

sd, standard deviation

Transcription of two genes involved in transport (*codB*, *yggB*) was affected. This group of genes also included some genes involved in sulfate such as a putative sulfatase gene (*b1498*) and a gene coding for a predicted anaerobic sulfatase maturation enzyme,

predicted DNA-binding transcriptional regulator b1497. These two genes may belong to the same transcriptional unit (data from http://biocyc.org/).

The specificically down-regulated genes in strain IHE3034 (see Table 18) included genes involved in motility and chemotaxism. *fliQ*, *fliF* were specifically down-regulated in IHE3034 and absent among the down-regulated genes of BEN374.

Table 18: Most down-regulated genes in human ExPEC strain IHE3034 at 41 °C versus 37 °C (p≤ 0.05)

Probe designation	SignificanceTest1535 :P Dataset:p value	M1 mean	M1 sd
fes	0.00386284563881326	-1.15266614029998	0.428977845155756
b1966	0.0011976961046789	-1.31821748074826	0.358886788469632
fepA	0.00343193512200591	-1.37306500084017	0.494819591040449
b1409	0.00129111920298342	-1.0197049508122	0.283144298498221
fliQ	7.18424993379995e-06	-1.29324159066566	0.0958330880869793
argT	0.0211219508414274	-1.17853621945433	0.715252597945729
nanT	0.0236263762774541	-1.22940835681496	0.772616122002189
fliF	0.000883922212047538	-1.20917012525715	0.304073075829504
oppD	0.00592653412209246	-1.01352954923035	0.424429545063899
ompC	0.000771816420381955	-1.60642288303423	0.389968612456984
ilvE	0.0193207355291711	-1.00771834469349	0.595034788187895
yhaO	3.19243761481044e-05	-1.06051894981428	0.114332929074428
yeeY	0.0195227757218422	-1.14631235312373	0.679032405978833
proX	0.00523348380164017	-1.16043885611988	0.469449233522207
cstA	0.0321119873115126	-1.20179779833286	0.833139514211038
b0805	0.00123427396838618	-1.55897805360676	0.427797382562192
b2862	0.00647610328459191	-1.02491540129356	0.439966404129324
yjiY	0.0378351616725445	-1.50185173943065	1.09918639158887
tsx	0.020406266348883	-1.00639965516503	0.604318139550193
cspA	0.0250851851670845	-1.33665228715835	0.855997014867116
cspE	0.0417738493782889	-1.0758194098531	0.814162981296411
ilvC	0.00317505182314715	-1.00371315378675	0.354169404906301
IHE3034_1_*CDS 89_0_0*	0.0379643428356921	-1.05514668224678	0.773131167390603
dgoA	0.0259397558264108	-1.4510698966484	0.939174554840198
b2809	0.00783762772373267	-1.51349240492987	0.685592747607731
fliY	0.00340502298195746	-1.15196255182291	0.414253775930393
yagU	0.0462922159546442	-1.01675962204753	0.797116147299299

sd, standard deviation

The fact that these genes have not been detected to be deregulated in the avian strain may be only due to the difference of quality in experiments and thus in statistics in case of human strain IHE3034. Additionally, some of these genes may be absent from the genome of strain BEN374 and could thus not be detected upon transcriptome comparison. A significant amount of genes involved in transport were specifically down-regulated (*fepA*, *argT*, *nanT*, *oppD*, *ompC*, *yhaO*, *proX*, *cstA*, *tsx*). Putative transporter or receptor were down-regulated, too (*b1966*, *b0805*). The *cspA* and *cspE* genes coding for regulator and the predicted regulator *yeeY*, were down regulated in this strain. Other genes are part of different metabolism pathways (*fes*, *ilvE*, *ilvC dgoA*). Their involvement in pathogenicity at 37 °C or 41°C should be analysed. *yagU*, an inner membrane protein that contributes to acid resistance was down-regulated.

A difference in some genes specifically affected in the human and avian strain has been observed. These genes code for regulators or putative regulators and predominantly for transporters and/or membrane receptors. Whether these differences, in gene expression, are due to pathotype-specific regulatory responses in the human or avian strain background, respectively or whether they result from their individual backgrounds, remains to be investigated.

5.1.6 Confirmation of microarray data by real-time RT-PCR

To confirm the microarray data, the transcript level of interesting candidate genes of each strain was quantified by real-time RT-PCR (see figure 19). The reduced transcription of selected genes representative of components of the flagellar apparatus and chemotaxis, i.e. *cheY*, *cheZ*, *aer*, *tar*, *fliA* and *fliC* at 41 °C was confirmed by real-time RT-PCR. This response to temperature elevation is well known (2, 201). Other genes of interest were analysed as well. The *hchA* (or *yedU*) gene which encodes the heat shock protein YedU (or Hsp31) was the only heat shock gene found to be induced upon temperature increase from 37 °C to 41 °C in the human and the avian strain. The *pyrI* gene was upregulated, too, under this condition in the human strain only. In case of the avian strain BEN374, *hdeA* and *hdeB*, which play a role in acid resistance, were

upregulated 8- and 10-fold, respectively. The gene *cadA* was up-regulated only in the avian strain at 41 °C.

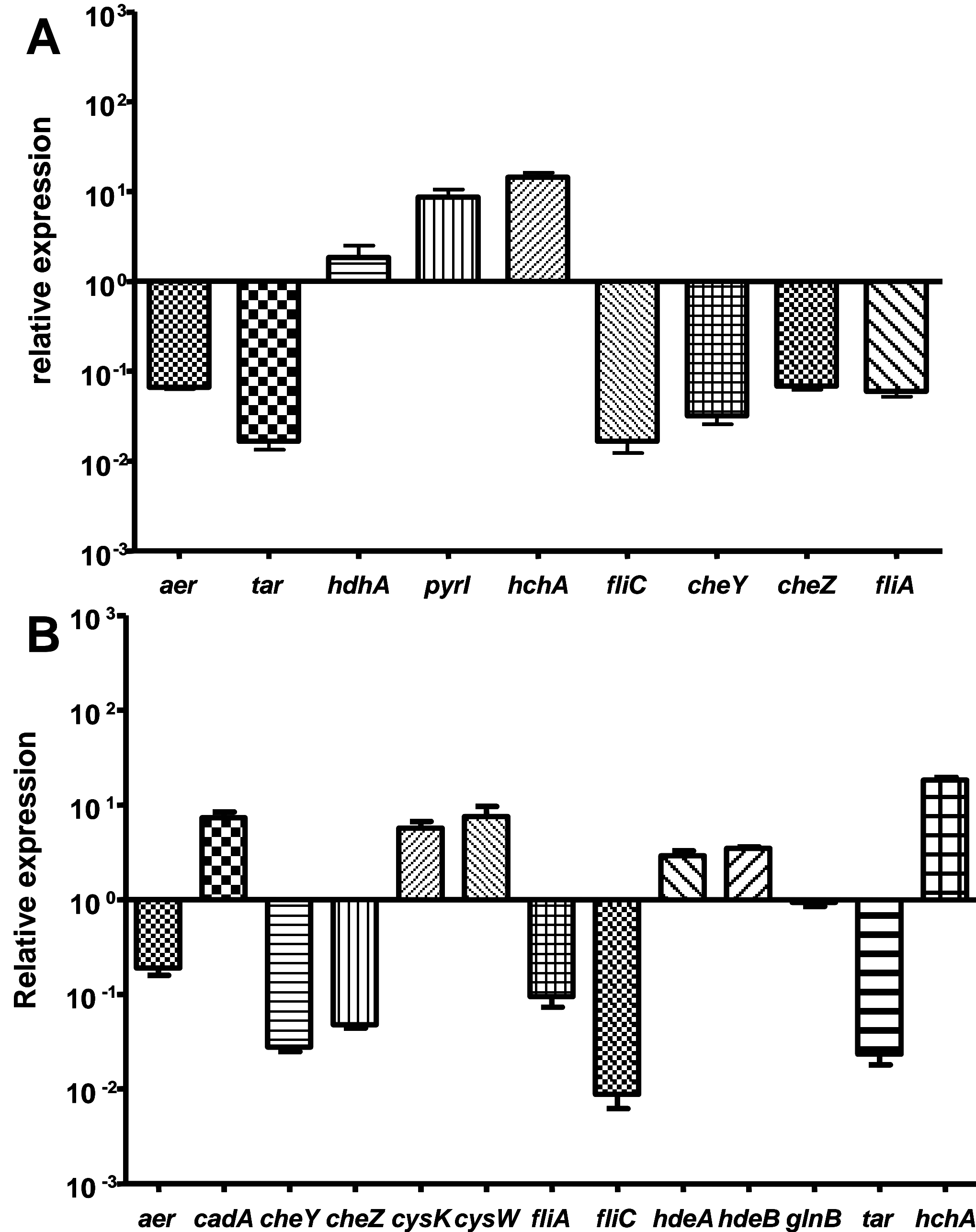


Figure 19: Validation of a set of temperature-regulated genes in *E. coli* IHE3034 A) and *E. coli* BEN374 B) by qRT-PCR. To confirm the microarray-based transcriptome data for IHE3034 and BEN374, both strains were grown in LB medium at 37 °C and 41 °C, respectively. Bacteria were harvested for RNA preparation at OD_{600} 0.6. Transcript levels of the indicated genes were quantified by qRT-PCR using equal quantities of RNA samples. The data was normalized using rRNA *frr*, as a reference, and graphed as the ratio of transcript levels at 37 °C vs 41 °C. Each value is representative of at least three different experiments with a p value < 0.05.

All flagella genes are down-regulated at 41 °C. Genes coding for factors involved in chemotaxis signaling (*aer*, *tar*, *cheY*, *cheZ*) were downregulated by factor 14, 50, 33, and 14, respectively. No difference in transcript levels could be seen for *flhDC*, indicating that the de-regulation of the flagella and chemotaxis genes was independent of the FlhDC master regulator. The *fliA* and *fliC* genes which are directly under the control of the FlhDC complex were downregulated by factor 16 and 62, respectively.

To assess if these genes are involved in virulence, *in vivo* competition assays were performed in 3-5 weeks old chickens (P. Germon, INRA Nouzilly). The wild type strain BEN374 and its isogenic mutant were used for infection of chickens by inoculation of the airsac. Under this condition, differences in virulence between the wild type BEN374 and the mutant BEN374*ΔhchA* could not be observed.

Furthermore, the expression of additional candidate genes was analyzed by real-time RT-PCR in other APEC strains grown at 37 °C and 41 °C. Serotype O2:K1 isolates of human (IHE3072) or avian (BEN2908) origins were chosen. In order to determine if the overexpression of the cystein operon *cysADJHKWN*, in *E. coli* BEN374 was strain-specific or pathotype-specific, *cysW* and *cysK* transcript levels were investigated in human isolate IHE3034 (see Figure 20).

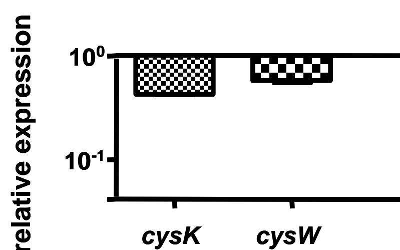


Figure 20: Relative quantification of the *cysK* and *cysW* transcript levels in IHE3034 at 41 °C versus 37 °C. Bacteria were harvested for RNA preparation at OD_{600}=0.6. Transcript levels of the indicated genes were quantified by qRT-PCR using equal quantities of RNA samples. The data was normalized using RNA of *frr* as a reference, and graphed as the ratio of transcript levels at 37 °C vs 41 °C. Each value is representative of at least three different experiments with a p value < 0.05.

The *cysW* transcript levels differed not significantly at 37 °C and 41 °C, respectively. Based on the results obtained for these two strains, we can see that there is

no difference of expression in the ExPEC strains above. The results obtained in BEN374 seem to be strain-specific and not linked to the avian or human origin.

Similarly, expression of the *cadA* gene was studied. Yet, as in the avian O18:K1 isolate BEN374, the *cadA* gene was found to be induced in *E. coli* strains IHE3072 and BEN2908 at 37 °C relative to 41 °C as well. However, if one applies a cut off-factor of 2, the expression level in the human isolate IHE3072 was at the limit of this cut off. In contrast, in *E. coli* BEN2908, *cadA* was overexpressed at least 10-fold, five times more than in the human ExPEC strain.

To assess if this difference in increased *cadA* transcription was strain-specific or not, *cadA* transcript levels were tested in other ExPEC strains of human and avian origin by real-time RT-PCR. Of the six strains tested (representing one human and one corresponding avian isolate of three different serotypes), all of them exhibited an increased *cadA* transcript level at 41 °C relative to 37 °C (Figure 21).

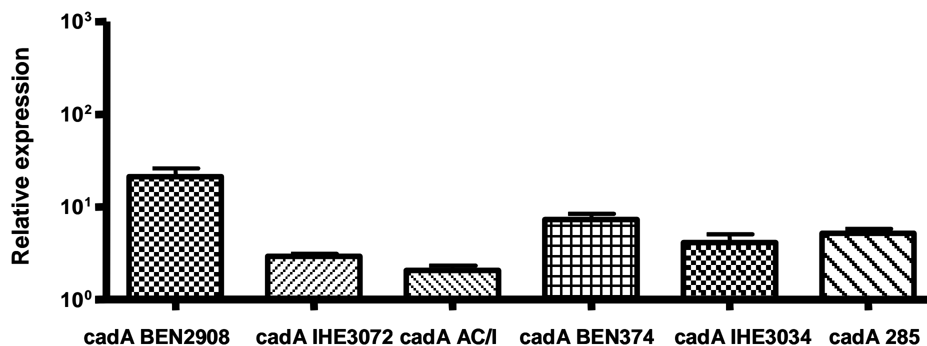


Figure 21: Analysis of *cadA* expression in avian and human ExPEC isolates at 37 °C vs. 42 °C. Strains were grown in LB medium at 37 °C and 41 °C, respectively. Bacteria were harvested for RNA preparation at OD_{600} 0.6. Transcript levels of the indicated genes were quantified by qRT-PCR using equal quantities of RNA samples. The data was normalized using the *frr* as a reference, and graphed as the ratio of transcript levels at 37 °C vs 41 °C. Each value is representative of three different experiments with a p value < 0.05.

With one exception (avian O78 isolate AC/1), the amount of *cadA* transcripts was always higher in avian isolates compared to human strains. Whether this increased induction of *cadA* transcription upon at 41 °C is biologically relevant, requires further investigation.

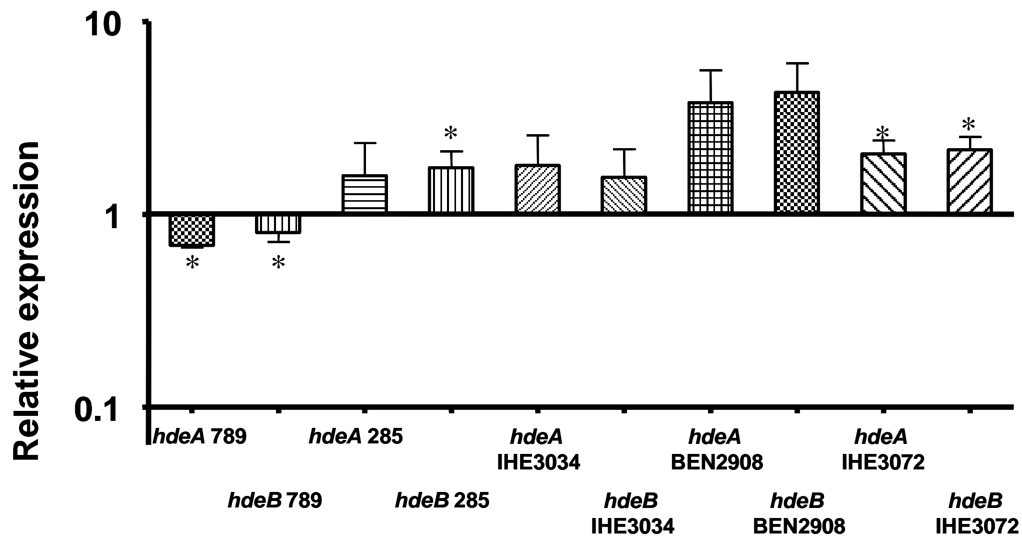


Figure 22: Analysis of *hdeA* and *hdeB* expression in avian and human ExPEC isolates at 37 °C vs. 42 °C. Strains were grown in LB medium at 37 °C and 41 °C, respectively. Bacteria were harvested for RNA preparation at OD_{600}=0.6. Transcript levels of the indicated genes were quantified by qRT-PCR using equal quantities of RNA samples. The data was normalized using the *frr* gene transcript as a reference, and graphed as the ratio of transcript levels at 37 °C vs 41 °C. Each value is representative of three different experiments. The * represents data with a p value < 0.05.

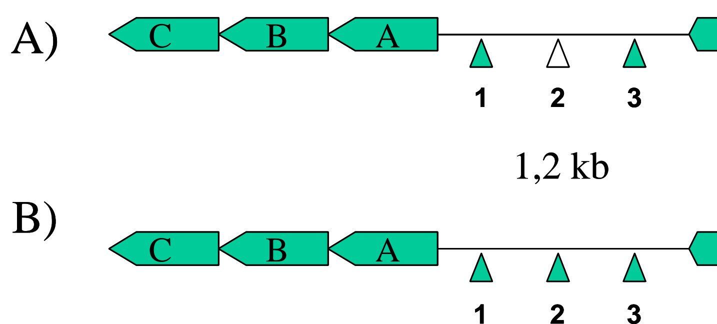
The *hdeA* and *hdeB* transcript levels were also checked in IHE3034 due to their role in acid resistance, despite their absence among the list of up-regulated genes identified by the microarray hybridizations (see appendix) due to its role for acid resistance. Surprisingly, the *hdeA* and *hdeB* genes were also up-regulated in IHE3034 at 41 °C. A significant upregulation of *hdeA* transcript levels in BEN374 upon growth at 41 °C could not be significantly confirmed by real-time RT PCR in all avian APEC (see Figure 22).

5.2: Analysis of Mat fimbriae expression in newborn meningitis *E. coli* isolate IHE3034

5.2.1 MatA expression and *in silico* promotor studies

To analyse the molecular mechanism of serotype-specific expression of Mat fimbriae which are only expressed in O18:K1 strains at 20 °C in LB medium, regulation of *matA* expression was studied. The *matA* gene codes for a regulatory protein which is required for expression of *matB*. Northern blot analysis of *matA* transcripts was not successful so far. The extremely low amount of *matA* transcripts may account for these difficulties. Alternatively, first attempts to quantify *matA* transcript levels by Real-Time RT-PCR indicated that transcript levels are higher at low growth temperatures, which confirms the phenotypic observation that Mat fimbriae are produced at 20 °C.

The putative promoter region of *matA* in strain IHE3034 was also investigated and compared with the corresponding DNA stretch of K-12 strain MG1655. Although the DNA sequence of the *matA* upstream region of these strains is not identical, three different putative promoters which could direct *matA* transcription could be detected by *in silico* analysis (see figure 23).



Predicted promoter with Transcriptor Factor (TF) binding sites

Figure 23: *In silico* analysis of *matA* promoter with Bprom (softberry software) in A) MG1655 and B) IHE3034. The putative promoters found in the CDS of *matA* were excluded from the figures. Putative TF binding sites for each promoter in IHE3034: **1**- RpoS17, **2**- Fis, PhoB, RpoD17, Fis, LexA, **3**- RpoD16, ArgR, ArcA; in MG1655: **1**- RpoD16 **2**- no such sites for this promoter **3**- RpoD16, ArgR , ArcA.

The putative promoters 1, 2 and 3 are highly similar between the two strains, but minor differences are present due to TF binding sites, nucleotide sequences or position (due to gaps between the promoters). Only the -10 and -35 signals of putative promoter 1 are identical between the MG1655 and IHE3034 strains (but not at the same distance of the CDS), but not for the putative TF binding sites. Promoter 2 does not possess known putative TF binding sites in case of strain MG1655. For more details, see appendix.

A transcript covering the region between putative promoter 2 and *matA*, but not between putative promoter 3 and *matA* could be amplified by RT-PCR. The *matA* promoter region of strain IHE3034 covering the *matA* gene and its upstream region including putative promoters 1, 2 and 3 was subcloned into the high copy vector pGEM-Teasy.

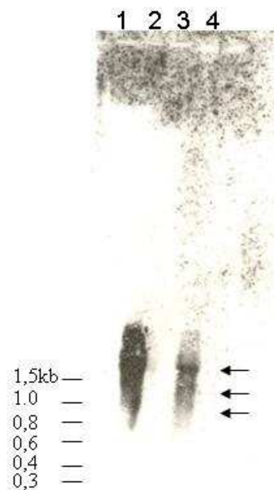


Figure 24: Northern Blot analysis with a promoter 2-specific probe and the following *E. coli* strains: 1: IHE3034-pGEMT*matA* and intergenic region 20°C, 2: IHE3034-pGEMT 20°C, 3: IHE3034-pGEMT *matA* and intergenic region 37°C, 4: IHE3034-pGEMT 37°C.

Northern blot analysis allowed the detection of at least three transcripts that comprise at least a part of the upstream region (see Figure 24). The Northern blot also confirmed that transcription of *matA* and/or of this upstream region was stronger at 20 °C than at 37°C.

5.2.2 Transcriptional organisation of the *mat* operon

Poutu *et al.* demonstrated that *matB* and *matC* were co-transcribed, but they couldn't prove that this was also the case with *matA*. A bioinformatical analysis did not reveal marked transcription start points for *matB* in the upstream region or in the *matA* gene. In addition, previous studies to determine the transcriptional start of *matB* by primer extension resulted in the sequences that ended upstream of the *matA* gene (data not show). Northern blots with a *matA* probe revealed a transcript of more than 2 kb in size, which corresponds not only to the size of the *matA* gene.

In order to characterise the transcriptional organization of *mat* operon, RT-PCRs were performed with RNA retrieved from bacterial cultures grown under conditions when Mat fimbriae were expressed. After growth in LB medium at 20 °C, to an OD_{600} of 0.6, total RNA was isolated and purified to be use in reverse transcription. To determine the transcription units of the *mat* gene cluster, the coding sequence was subjected to *in silico* analyses and RT-PCRs were performed. The results obtained demonstrate that the *mat* determinant is divided into two putative transcriptional units: the genes *matABCD* are located in the same reading frame, and also the *matEF* genes are located in another reading frame. The results are summarised in Figure 25.

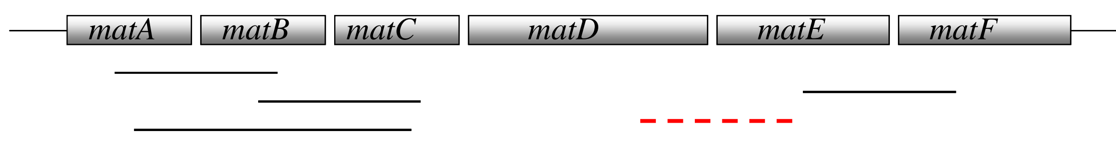


Figure 25: Transcriptional organisation of the Mat fimbriae gene cluster of *E. coli* strain IHE3034. This scheme represents the results of RT-PCR experiments performed on the *mat* operon of *E. coli* IHE3034. No RT-PCR products could be amplified between *matD* and *matE*. The experiments were performed with 1 or 2 µg of RNA extracted from a culture grown at 20 °C in LB medium and 220 rpm, until it reached the mid-log growth phase.

The region between *matA* to *matD* could be amplified from mRNA and the amplified fragment size of the PCR products correspond to the theoretical expected size between *matA* and *matD* i.e. including the *matB* and *matC* sizes. It was the same for the product between *matA* and *matB*, *matA* and *matC*, and, finally, between *matE* and *matF*. These results confirm the *in silico* analysis of the *matABCDEF* DNA sequence.

5.2.3 Analysis of *matA* transcription

To determine the transcription start point of *matA*, primer extension analysis was performed. In view of the results (smear due to degradation of the *mat* transcripts), we used another method to determine the transcriptional start, i.e. by 5'-RACE. For this purpose, RNA was extracted from strain IHE3034 grown to mid-log phase at 20 °C in LB medium with agitation (220 rpm). The results are present and summarize in the figure 26.

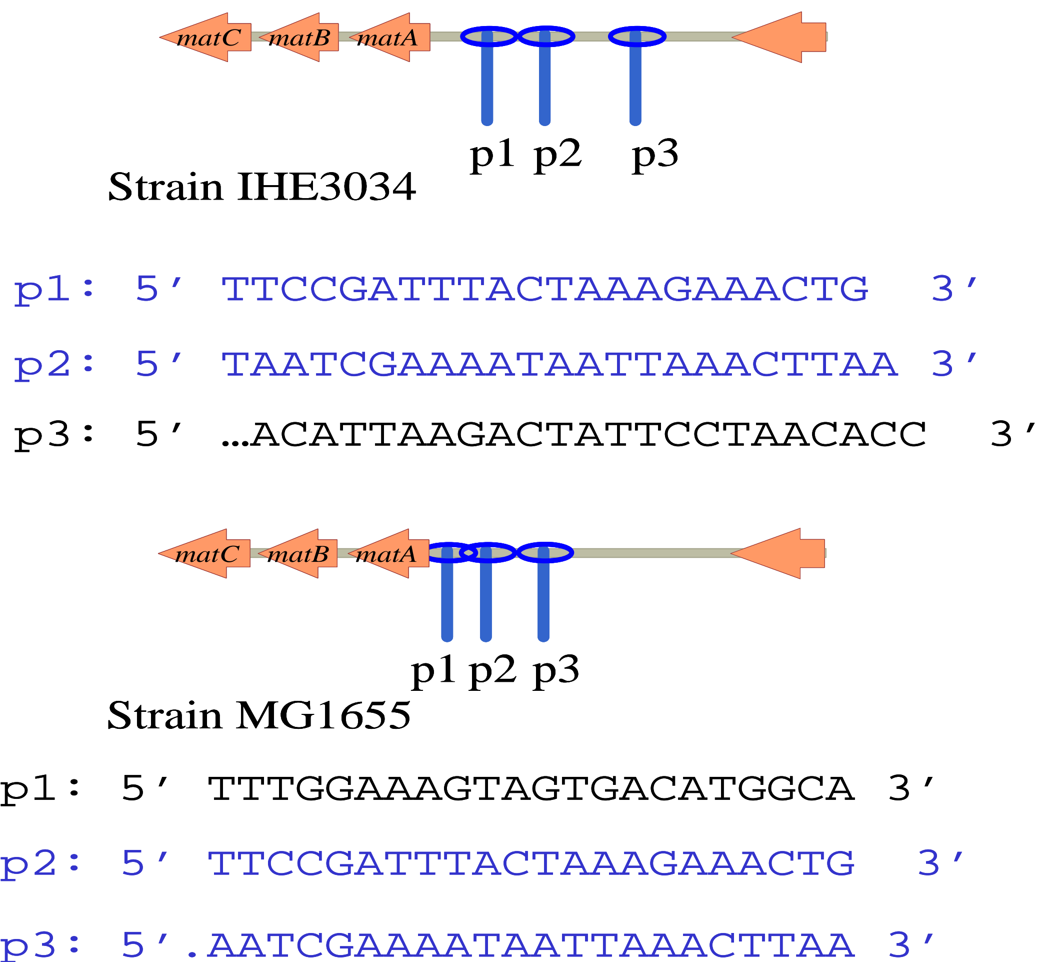


Figure 26: Transcriptional start point of *matA* in *E. coli* strains IHE3034 and MG1655. The transcriptional starts detected in both, *E. coli* strains IHE3034 and MG1655 are indicated in blue. Specific transcriptional start points only detected in one strain are shown in black. These data result from at least three independent experiments for each strain. Variable start positions of DNA sequence obtained from the 5' RACE analysis are indicated by points.

In strain IHE3034, three transcription start points were detected in a region of about 300 bp upstream of *matA*. Whereas the first and second start points could be clearly identified, the exact start of the third one could not be unambiguously determined, probably because of the instability of the extreme 5'-end of the mRNA.

In K-12 strain MG1655, three transcription start points exist as well, which are relatively closely located to each other (in a region of about 100 bp upstream of *matA*). Again, the first two start sites could be exactly identified, but not the third one which exhibited marked instability of its extreme 5'-end. The second transcriptional start point of *matA* was identical to the first one of strain IHE3034. Furthermore, the third *matA* transcriptional start, although it could not be exactly identified, was found to be almost identical to the second transcriptional start point of *matA* in strain IHE3034. The missing 5'-T in the 5'-*matA* transcript end of strain MG1655 relative to *E. coli* IHE3034 may be explained with less DNA sequences obtained with the 5'-RACE in case of the K-12 strain than for *E. coli* IHE3034.

5.2.4 Effect of H-NS on *mat* gene expression

This work was done in collaboration with T. Lehti (Biocentre, University of Helsinki). The strain IHE3034 *hns* was contructed by T. Lethi. To identify activators that could affect Mat fimbriae expression, Timo Lehti screened a Tn5 insertion library in *E. coli* IHE3034 to find mutants which had lost temperature-dependent expression of Mat fimbriae. Seven mutants were found in which the transposon was inserted at the identical site in *hns*. All these mutants expressed Mat fimbriae at 37 °C, unlike the wt IHE3034. For complementation, the *hns* gene of *E. coli* IHE3034 was cloned with its upstream region into pBR322.

As H-NS represses several virulence functions induced by environmental signals through action of specific activators and our hypothesis is that MatA functions to induce Mat fimbria expression repressed by H-NS. To understand the differential expression of Mat fimbriae in *E. coli* IHE3034 and IHE3034 *hns* as well as the mechanism by which

MatA and H-NS regulate Mat fimbriae expression, we quantified transcript levels of the *mat* genes by real-time RT-PCR. Strains IHE3034 and IHE3034 *hns* were grown at 20 °C and 37 °C in LB medium to mid-log growth phase and the RNA was extracted. Real-time RT-PCR analysis revealed that transcript levels of all the genes of the *mat* determinant were up-regulated in the *hns* mutant relative to the wild type (see Figure 27).

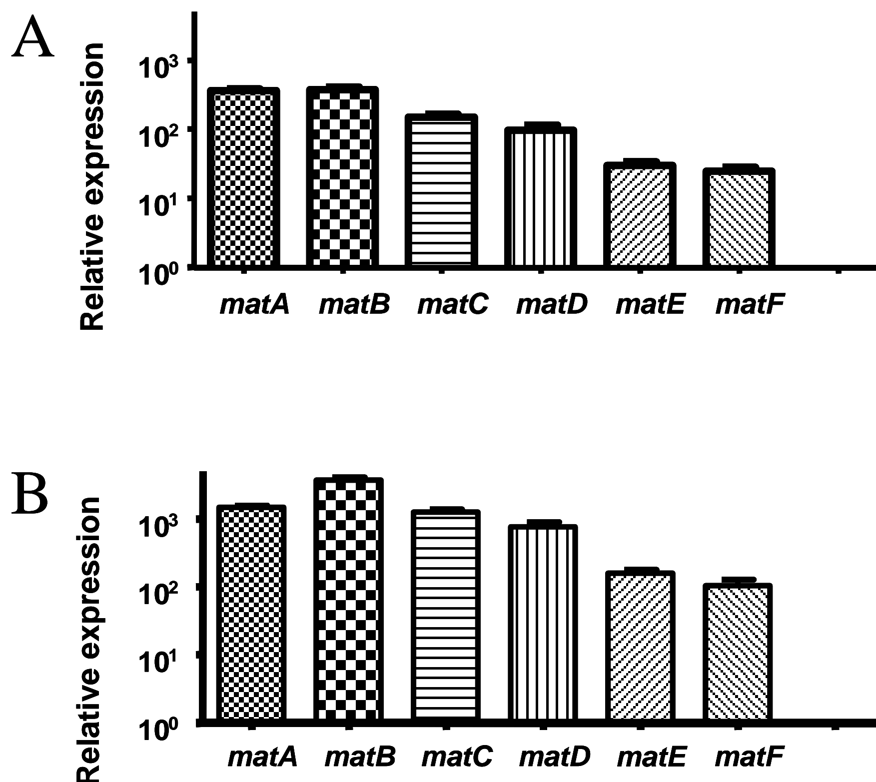


Figure 27: Comparison of transcript levels of the different *mat* genes in *E. coli* IHE3034 *hns* relative to its wild type strain at 20 °C and 37 °C. The real-time RT-PCR experiments were performed in the strain IHE3034 *hns*::Tn*5* and wild type strain IHE3034, in LB medium under shaking conditions (220 rpm). The figure A represents the relative expression at 37 °C, when Mat fimbriae are not expressed in wild type strain IHE3034. Figure B represents the results obtained at 20 °C, when Mat fimbriae are also expressed in strain IHE3034. The data depicted represent the mean values of at least three independent experiments, $p \leq 0.05$.

These data suggest that H-NS represses expression of Mat fimbriae at 20 °C and 37 °C. At 20 ºC, Mat fimbriae were strongly expressed in strain IHE3034 *hns* in the presence of *matA*. This was also observed at 37 ºC. Obviously, Mat fimbria expression is regulated by *hns* independently of the temperature-dependent mechanism which favours Mat fimbriae production at low temperatures. It was concluded that at 20 ºC MatA

functions as an H-NS antagonist and at 37 ºC counteracts repression by an unknown mechanism. H-NS represses gene expression by binding upstream and/or downstream of promoter regions hence hindering RNA polymerase function.

5.2.5 Transcriptome comparison of *E. coli* IHE3034 and its *matA* mutant

Since studies on the MatA regulator and its potentials target sites have not been performed, a transcriptome analysis was conducted between IHE3034 and its isogenic mutant IHE3034 *matA*-H179P, which does not express Mat fimbriae. The transcriptome analysis allowed us to screen global transcript levels in strain IHE3034 and its *matA* mutant. The conditions under which the transcriptome analysis were performed allowed Mat fimbriae expression (growth in LB medium at 20 °C in a shaking culture at 200 rpm until the exponential phase has been reached). Transcriptional profiling was performed using custom made *E. coli* microarrays (Operon). At least four independent microarray hybridizations for each condition tested were performed, including a dye-switch. The hybridization was carried out at 42 °C for 20 hours. Each experiment was performed with RNA obtained from independent bacterial cultures.

All data (*matA* mutant vs. wild type) were therefore pooled, and after statistical analysis of the four independent experiments, only few genes were pertinently de-regulated. These repressed genes belonged to the same operon of the *mat* gene cluster including *matA, matB, matC* and *matD*. Other genes with significant MatA-dependent transcription could not be identified. MatA seems only to be the transcriptional activator of these four genes. We can see an autoregulation effect of *matA*, due to the fact the gene is still expressed in the mutant but not able to bind on DNA. Interestingly, *matE* and *matF*, which are on different transcripts, are not regulated by MatA.

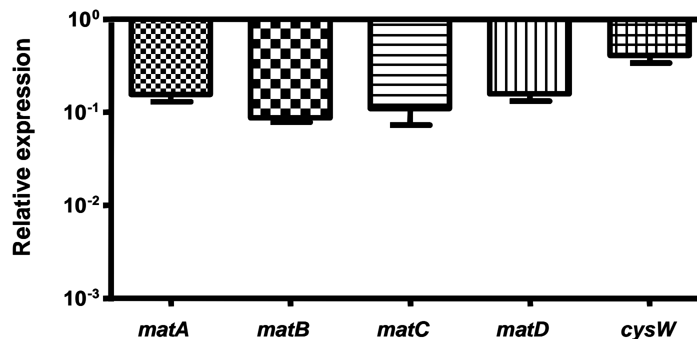


Figure 28: Comparison of transcript levels of the different *mat* genes and *cysW* in *E. coli* IHE *matA*-H179P relative to its wild type strain at 20 °C and 37 °C. The real-time RT-PCR experiments were performed in LB medium, grown under shaking conditions (220 rpm) until mid-log growth phase. The data depicted represent the mean values of at least three independent experiments, $p \leq 0.05$.

These data were confirmed by real-time RT-PCR experiments (see Figure 28) and thus proved the role of MatA as an activator of the Mat fimbriae expression, precisely on *matA*, *matB*, *matC* and *matD*. But in case of *cysW*, this gene appeared to be down-regulated.

5.2.6 Sequence analysis of *matA* and *matB*

The gene cluster coding for Mat fimbriae is widely distributed among pathogenic and non-pathogenic *E. coli* strains. To verify, if like in case of other fimbrial determinants, amino acid sequence variability may be detected in case of the putative adhesin subunit of Mat fimbriae, the MatB protein sequence was compared among the strains of the COLIRISK strain collection. The *matB* gene of the individual strains tested displayed several variable nucleotide positions (see appendix). When the deduced amino acid sequences were compared (see Figure 29), allelic variation of this adhesin could be observed. Unfortunately, in the absence of a known interaction partner of the MatB protein, it is difficult to analyze the effect of these variations on the secondary structure of MatB which may affect this protein's receptor specificity.

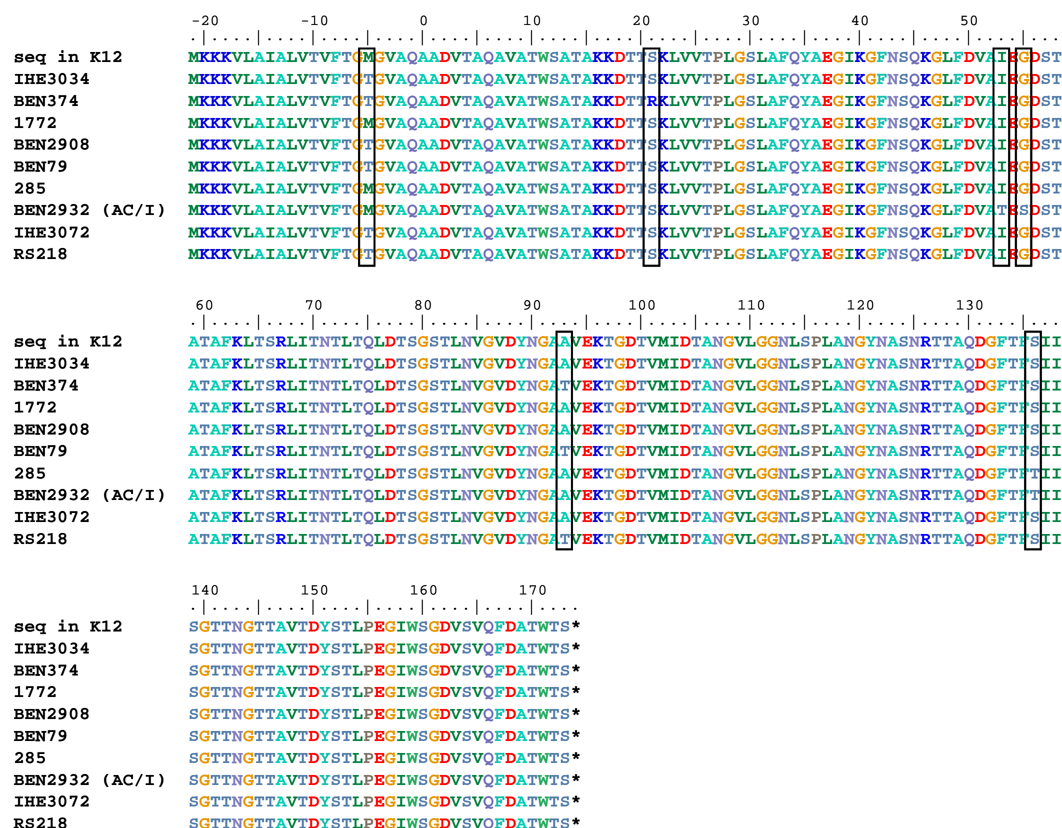


Figure 29: Amino acid sequence of the MatB protein in the *E. coli* strains of the COLIRISK strain collection. Allelic variations of the MatB protein in the different *E. coli* strains are boxed (S21R; I53T; G55S; A93T; S136T). The substitution positions are boxed (S21R; I53T; G55S; A93T. Mutation in the N-terminal signal: T-6M).

The alignment of all MatB protein sequences from the COLIRISK strain collection displays some variability in the amino acid sequence. Their effect on the binding must be characterized in the future.

With regard to the specific conditions of Mat fimbriae expression, the *matA* nucleotide sequence and the MatA protein sequence were also analyzed (see Figure 30). In the entire protein sequence, seven amino acid variations could be detected in this regulatory protein.

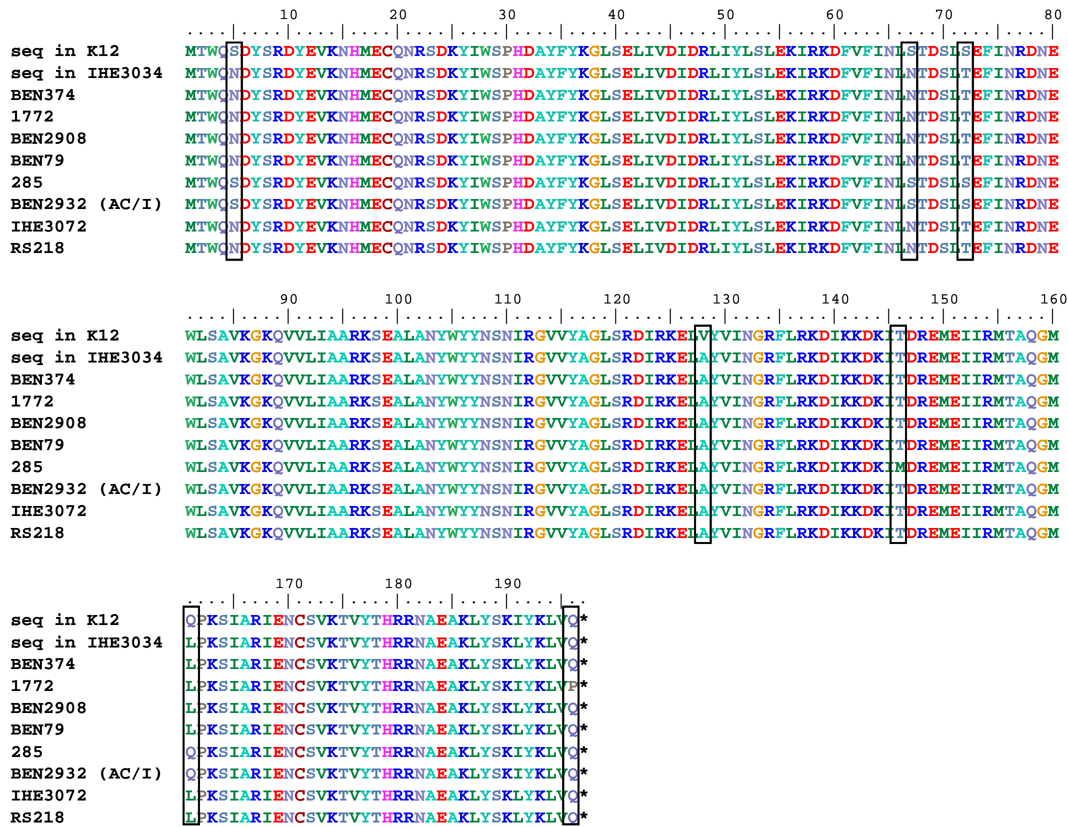


Figure 30: Amino acid sequence of the MatA protein in the *E. coli* strains of the COLIRISK strain collection.

The protein MatA is encoded by the first gene of the *mat* determinant. The MatA sequence comprises 196 amino acids, lacks a detectable signal sequence, and contains between the residues 125 to 195 a predicted helix-turn-helix DNA-binding motif similar to the consensus pattern in the LuxR family of regulatory protein. Allelic variations between the different isolates of the COLIRISK collection (S5N, S67N, S72N, V128A) and four amino acids exchanges in the helix-turn-helix DNA-binding motif (V128A , T146M, Q161L, Q196P) are boxed. The alignment of all MatA regulator protein sequences from the COLIRISK strain collection displays some variability in the amino acid sequence. Their effect on the Mat fimbriae expression must be characterized in the future.

5.2.7 Biofilm formation

The ability of bacterial cells to bind to abiotic surfaces, the surface of eukaryotic cells, as well as to express specific extracellular matrix substances, can result in the formation of multicellular bacterial communities (biofilm). One prerequisite for the formation of biofilm is direct cell-to-cell interaction which needs expression of definite factors - fimbrial and non-fimbrial adhesins, surface proteins, and extracellular matrix polymers (314). The expression of type 1- and curli fimbriae is known to contribute to the ability of *E. coli* to form biofilm, as well as cellulose biosynthesis, flagella, colanic acid and Ag43 expression (69, 102, 132, 284, 370). When one or more of these factors are expressed, bacteria tend to aggregate and form complex multicellular communities.

For strain IHE3034, a strong biofilm formation could be observed at low temperature (less than 30 °C) in LB or minimal medium, but not at 37 °C (see Figure 31). As Mat fimbriae expression occurs under the same conditions under which biofilms are formed, it has been investigated wether Mat fimbriae expression may contribute to biofilm formation.

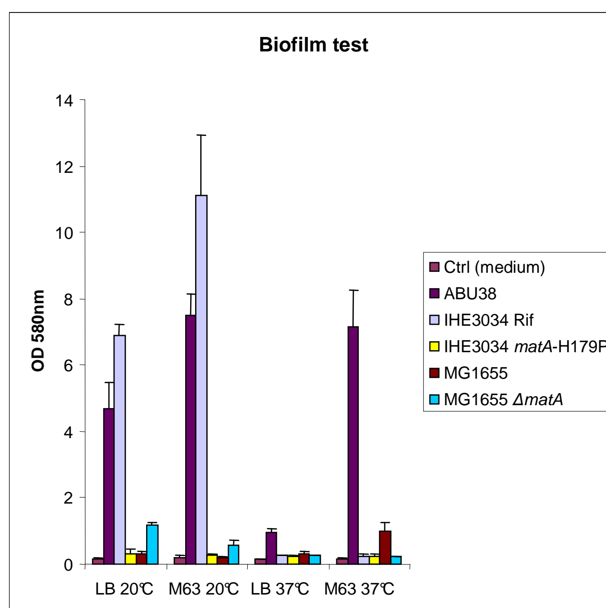


Figure 31: Mat fimbriae contribute to biofilm formation of *E. coli* strain IHE3034. Biofilm formation was tested at 20 °C and 37 °C in LB and M63 medium in microtiter plates. Strong biofilm formation occurred at 20 °C due to the presence of Mat fimbriae in *E. coli* IHE3034. ABU strain 38 was used as a positive control, and a well only filled with medium served as a negative control. The data depicted represent the mean values of at least three independent experiments, $p \leq 0.05$.

In order to identify whether Mat fimbriae were involved in biofilm formation, biofilm tests were performed in PVC 96-well plates at 20 °C for 48 h in LB and M63 medium. At 37 °C, biofilm formation was measured after 24 hours of growth. In this experiment, we used the strain IHE3034, its isogenic mutant IHE3034 *matA*-H179P, the mutant deleted for *matBCDEF* (IHE3034 *ΔmatBF*) which lacks the Mat fimbriae structural genes and the corresponding complemented mutants. The data presented in figure 32 clearly indicate a correlation between the presence of the functional *mat* gene cluster and biofilm formation. It was also demonstrated that only the Mat fimbrial organelle is involved in the biofilm formation. This is one of the first biologically relevant functions of this fimbrial type which could be clearly defined.

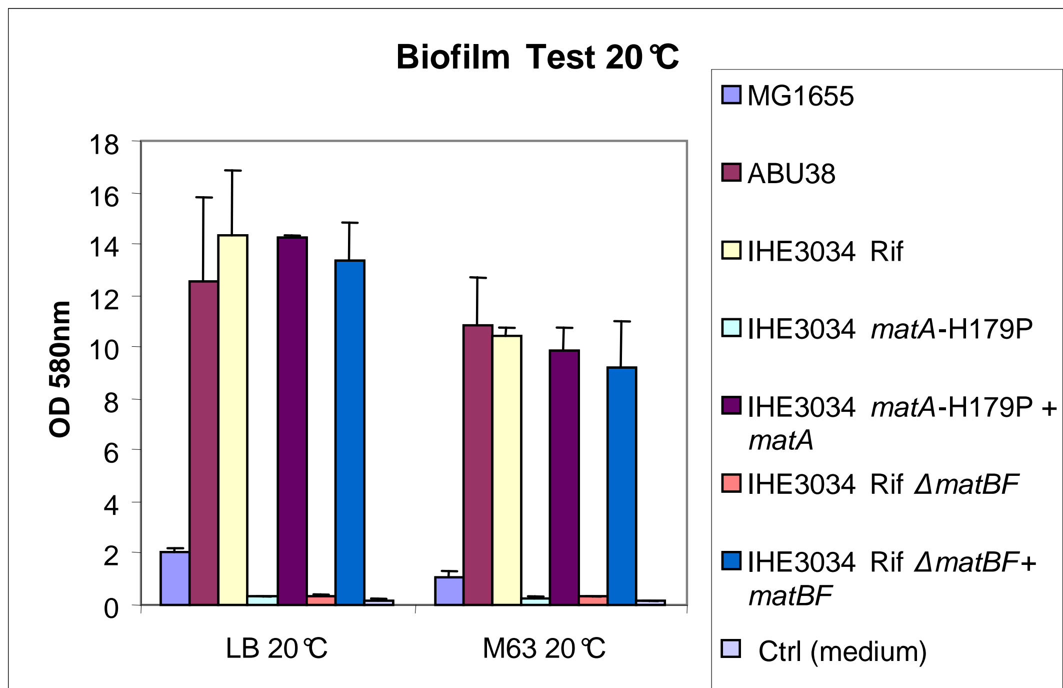


Figure 32: Analysis of the impact of MatA and the Mat fimbriae on biofilm formation of *E. coli* strain IHE3034. Biofilm test were performed at 20 °C in LB and M63 medium. Strong biofilm formation occurred at 20 °C in the presence of functional Mat fimbriae in IHE3034. The strain IHE3034 *ΔmatBF* expressed MatA but not the Mat fimbrial organelle. The ABU strain 38 was used as a positive control and a well only filled with medium served as a negative control. The data depicted represent the mean values of at least three independent experiments, $p \leq 0.05$.

5.2.8 Phenotypic Analysis of *E. coli* strain IHE3034 and its Mat fimbriae mutant

The phenotypes of the *matA* mutant of *E. coli* strain IHE3034 were tested by classical physiological assays for typical virulence traits of extraintestinal pathogenic *E. coli*. In case of type 1 fimbriae (*fim*), P-fimbriae and S-fimbriae (*sfa*), their expression was not affected in the *matA* mutant. This supported the microarray results. Other virulence characteristics like haemolytic activity, or iron uptake have been checked. These traits were, if present, not affected in *E. coli* IHE3034 by the inactivation of MatA or affected by growth temperature.

5.2.8.1 Motility and chemotaxis

Biofilm formation and motility are often linked or interdependent. Accordingly, the expression of genes involved in motility and chemotaxis was analyzed by swimming tests on LB agar plates at 20 °C. The strain IHE3034 and its isogenic mutant IHE3034 *matA*-H179P were compared on their swimming abilities on LB agar plates with 0.3% agar, that have been inoculated from another plate or from overnight cultures adjusted to the same OD (Figure 33 A). The complemented mutants were usually less motile than the wild type. Comparable phenotypes have been observed with strain BEN374 as well as its mutants BEN374 *ΔmatA* and BEN374 *ΔmatB* (Figure 33 C).

Figure 33: Influence of Mat fimbriae expression on motility of *E. coli* strain IHE3034 and its *matA* mutant. The swimming ability was tested on 0.3 % agar plates. Representative results from more than three independent experiments are shown. A) Motility of strain IHE3034, its isogenic mutant IHE3034 *matA*-H179P and the complemented mutant IHE3034 *matA*-H179P/*matA*, at 20 °C after 48 h of incubation. B) Motility of strain IHE3034, its mutant IHE3034 *ΔmatBCDEF* and the complemented IHE3034 *ΔmatBCDEF/matBCDEF*. C) Motility of strain BEN374 which expresses Mat fimbriae at a lower level than IHE3034, its mutant BEN374 *ΔmatA* and BEN374 *ΔmatB*, at 20 °C for 48 h.

This reproducible experiment is a clear evidence for an effect of the *matA* expression on the strain's motility. In the absence of a functional *matA*, motility was markedly increased. The molecular reason for this phenotype could not be characterized so far by real-time PCR or microarray hybridization. According to the available data, *matA* is expressed under the experimental conditions used. We could also show that Mat fimbriae were not expressed in K-12 strain MG1655 under all conditions tested. But when *matA* was deleted, the same effect appeared in this strain, too (Figure 34).

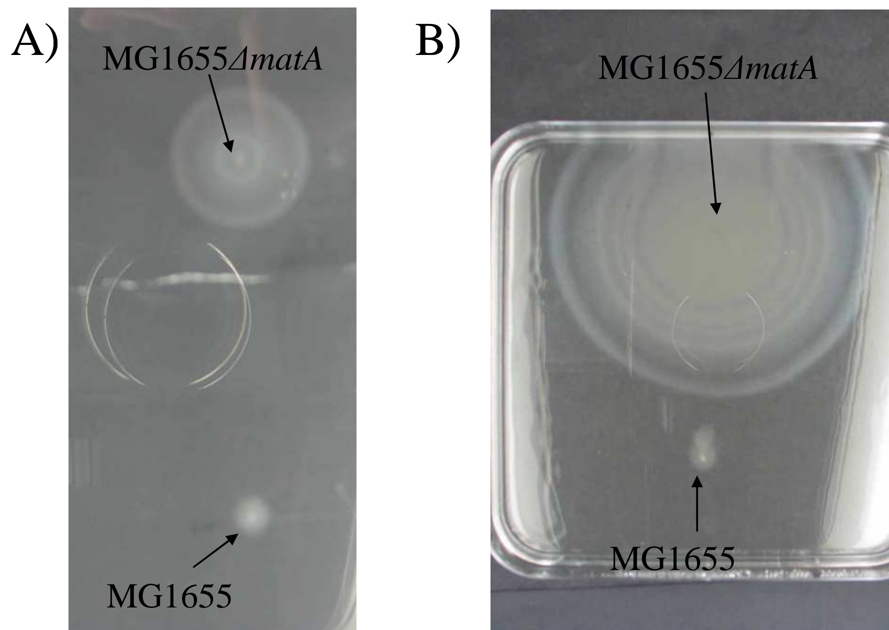


Figure 34: Influence of MatA on motility of *E. coli* strain MG1655. The motility tests were done on 0.3 % agar plates. Representative results from more than three independent experiments are shown. The motility of strain MG1655 and its mutant MG1655 *ΔmatA* was tested after 48 h of growth at 20 °C A) and at 16 h of growth at 37 °C B).

Until now, it is unknown in which way the motility is increased and how MatA may play a role in its regulation.

To decipher why this difference was not detectable at the gene expression level from the microarrays data, real-time RT-PCR experiments (see Figure 35) were performed on genes involved in motility like *fliA* and *fliC*.

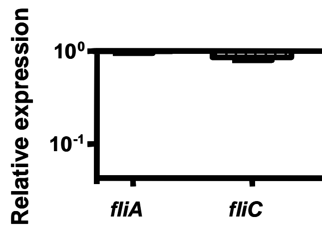


Figure 35: Comparison of transcript levels of the *fliA* and *fliC* genes in *E. coli* IHE3034 *matA*-H179P relative to its wild type strain at 20 °C. The real-time RT-PCR experiments were performed in LB medium, grown under shaking conditions (220 rpm) until mid-log growth phase. The data depicted represent the mean values of at least three independent experiments ($p \leq 0.05$).

The results indicated no significant differences in their expression and confirmed the fact they didn't appear to be de-regulated according to the microarray experiments. Thus, this motility increase has to rely on another mechanism.

5.2.8.2 Virulence effect in animal models.

The contribution of Mat fimbriae to *in vivo* virulence of *Escherichia coli* was compared using a *matA* and a *matBCDEF* deletion mutant of avian pathogenic *E. coli* strain BEN374. The mutant and wild type strain were tested in chickens by inoculation of the airsac. This experiment was performed in collaboration with P. Germon (INRA, Nouzilly). According to the results obtained, Mat fimbriae did not significantly affect *in vivo* virulence of APEC strain BEN374.

6. Discussion

6.1 The potential zoonotic risk of avian pathogenic *E. coli*

In this study, the role of temperature-dependent differences in gene expression of *E. coli* strains that can cause systemic infection in humans and avians, i.e. human ExPEC and APEC isolates was explored. The aim was to answer the question whether host specificity of human ExPEC and APEC exists and if this may involve individual gene expression patterns as these pathogenic variants are frequently closely related and exhibit very similar genotypes. These data should also be helpful to evaluate the potential zoonotic risk between such avian and human extraintestinal pathogenic *E. coli* isolates, avian host may be a reservoir for human ExPEC.

Since the body temperature is one of the important differences between humans (37 °C) and chickens (41 °C), this predominant factor may affect gene expression of the colonizing bacteria. Our understanding should focus on the differential expression of genes coding for known or putative virulence and fitness-associated factors. Therefore, a closer look at the genes present in human and chicken isolates, but de-regulated at the body temperature of their corresponding host, should help to determine their impact on virulence or fitness under the two different growth conditions and indicate a potential zoonotic risk.

6.1.1 Absence of a specific genotype for human and avian ExPEC

To decipher differences between human ExPEC and APEC, a lot of studies have been performed to compare a plethora of isolates from human or avian sources with the aim to find specific virulence factors, metabolism pathways or structural features for one or the other group of isolates (98, 99, 167, 222, 285). A correlation between the presence of specific factors and the origin of isolates could not be clearly identified so far. Ron and co-workers showed a variable profile of virulence genes as well as the presence of mobility related sequences, pointing to the existence of a “mix and match” combinatorial

system (220). With the fast development of genomic analyses, nucleotide sequences of plasmids and genomes became available and, although not always published, did not reveal a specific genotype for human ExPEC and APEC (159). Another recent study indicates, however, that highly virulent APEC variants may be distinguished from less virulent isolates on the basis of certain genes which are frequently located on colicin plasmids (161). Interestingly, comparison of an APEC O2 isolate carrying a wild type ColV plasmid with individual ColV plasmid mutants that lack known ExPEC virulence-associated genes such as *iss*, *tsh*, *iutA*, *iroN*, *sitA* and *cvaB* indicated that these mutants were as virulent as the wild type thus suggesting that there may be other compensatory virulence factors (301). As the distribution of virulence factors seems often to be independent of their host, ExPEC strains from the same host type can have different sets of virulence-related genes as well as isolates from different host types can share the same virulence-related determinants. Because of this, we selected strain pairs of relevant serotypes, where one isolate was of human and the other one of avian origin (Tab. 12). Thus, these pairs of strains should guarantee a similar genetic background for each serotype in order to exclude that the differences observed result from genomic differences rather than from a different regulation of gene expression at the transcriptional or post-transcriptional level. The comparison of global transcriptional profiles extends the current knowledge based on genotyping regarding the possible zoonotic risk due to ExPEC of human and avian origin.

6.1.2 Phenotypic differences between human ExPEC and APEC

Based on the genotypic characterization of human ExPEC and APEC (Tab. 11 and 12), both groups could not be clearly distinguished. The different routes of infection in humans and avians suggest that virulence factors shared between human and avian isolates may not be used in the same way.

Among these virulence factors, adhesins may be involved at different stages of the infection and in different sites of the body (62, 80, 186, 315). Three important types of fimbriae, i.e. type 1 fimbriae, P-family adhesins and S-family adhesins have so far

been described to be involved in ExPEC infection. During urinary tract infection, the role of type 1-, P- and S-family adhesins has been well characterized (25, 62, 122, 218). In case of avian infections, the presence and expression of these fimbriae have been already demonstrated (80, 259), but whether their role and importance, e.g. in tissue tropism, is different in avians and in humans has not yet been completely understood.

These fimbriae are factors contributing to the virulence potential of such strains, but they are not necessarily sufficient to cause disease (219). I used different specific antibodies raised against these adhesins to evaluate their expression in the human and avian ExPEC strains at different temperatures. The human ExPEC and APEC strains could not be differentiated based on the expression of type 1, P- and S-family adhesins (Tab. 12). In addition, expression of these fimbriae are subjected to phase variation, which quickly switches between the "on" and "off" state of expression under certain conditions *in vitro* (88, 105, 211, 224) and *in vivo* (123, 303, 304, 363).

Curli are factors contributing to the virulence potential of such strains, but they are not necessarily sufficient to cause disease in avian (113, 114) and human (242, 370). Curli fimbriae are predominantly expressed in human and avian isolates between 20 °C and 37 °C. The human ExPEC and APEC strains could not be differentiated based on the expression of curli between 37 °C and 41 °C.

Other virulence-associated factors like iron acquisition systems have been studied. It is well known that ExPEC can usually express more than one iron uptake system. Until now, up to six iron acquisition systems have been clearly identified (aerobactin, enterobactin, yersiniabactin, salmochelin, a hemin uptake system and the Sit (*Salmonella* iron transport system)) (25, 83, 113, 116, 153, 232, 281, 297) . Due to the importance of iron in the organism and the difficulty to supply sufficient amounts of this essential nutrient, ExPEC strains have developed multiple strategies to capture Fe^{3+} ions from different source and under different conditions (271). Nevertheless, the genotypic analysis of human and avian ExPEC did not display different iron uptake capabilities that could explain host specificity (Tab. 11). The iron acquisition systems are individually

expressed in each strain and no correlation between the presence of different siderophore systems, their temperature-dependent expression and the strain origin could be seen. The precise role of the host (avian or human) for expression of individual iron acquisition systems as well as the impact of differential siderophore expression during infection on host specificity could, however, not be addressed in this thesis.

6.1.3 Different gene expression patterns between Human and avian O18:K1 ExPEC isolates IHE3034 and BEN374

In this study, we concentrated on one of the major factors which may influence the transcriptome of ExPEC in the human and avian host: the different body temperature of humans (37 °C) and avians (41 °C) may result in different gene expression patterns of human and avian isolates. Alternatively, ExPEC strains may not specifically respond to the different growth temperature.

Only a few genes were induced at 41 °C in each strain relative to growth at 37 °C. In this group, only 5 genes were commonly upregulated in these two strains (Tab. 15 and 17). One promising candidate gene that could be involved in the virulence of ExPEC at 41 °C was the *yedU* (*hchA*) gene. This gene codes for the protein Hsp31, a homodimeric member of the ThiI/DJ-1/PfpI superfamily that combines molecular chaperone and aminopeptidase activities. Interestingly, this gene was the only heat shock protein overexpressed at 41 °C in both strains. The hypothesis that this chaperone may maintain the correct tertiary structure of proteins expressed at the avian body temperature, including virulence factors, was not supported by an *in vivo* infection experiment. Differences in virulence between APEC strain BEN374 and its isogenic mutant BEN374 *ΔhchA* could not be observed upon experimental infection of 3-day old chickens (P. Germon, Nouzilly, data not shown). If this heat shock protein plays a role for the proper folding of proteins and/or their half-life, this is not preponderant for the virulence of strain BEN374 in a chicken.

Other candidate genes (*hdeA*, *b0834*, PAI I *Orf 55*, ME EO 28C *rorf1.1*; see appendix) could not be directly linked with virulence. The *hdeA* gene was checked in IHE3034 due to its role for acid resistance. Surprisingly, the *hdeA* and *hdeB* genes were upregulated, too, in IHE3034 at 41 °C. A significant upregulation of *hdeA* transcript levels in BEN374 upon growth at 41 °C could not be confirmed by real-time RT PCR in all avian APEC at 41 °C. Whether acid resistance may contribute to *in vivo* virulence in the chicken would require further analysis in analyzing more strains and maybe by comparing *in vivo* virulence of a wild type versus a *hde* mutant. In case of the avian pathogenic *E. coli* strain, further studies concerning the acid proteins and acid resistance should be performed to see whether they may be involved in virulence or host specificity. Expression of the *cysADJHKWIN* gene cluster, which was upregulated at 41 °C in strain BEN374, was not differently expressed at human and avian body temperature in the human ExPEC strain IHE3034. The results obtained for *E. coli* BEN374 may be strain-specific and not linked to host specificity. However, a potential role for virulence in human and avian ExPEC cannot be excluded from the results. To determine whether the encoded proteins affect virulence, more ExPEC strains should be tested to see a preferential correlation between the expression of these genes in human ExPEC and/or in APEC. The increased transcription of the *pyrI* gene at 41 °C in strain IHE3034 was confirmed by real-time RT-PCR. Another gene, *map*, was strongly down-regulated in BEN374 and maybe a potential candidate for virulence. The *E. coli* MAP is a type-I enzyme and is a potential antibiotic target; selective inhibitors have been designed (321).

In strain IHE3034, less genes were found to be upregulated than in BEN374. This may be due to many factors, including strain-specific gene expression profiles as well as the fact that the probes spotted on the microarray mainly covered the genome of *E. coli* K-12 strain MG1655 and not the complete genome of both O18:K1 strains tested in this study.

Surprisingly, the number of the down regulated genes, more than 70 genes for each strain, was higher relative to the number of upregulated genes. This group of genes mainly contained genes involved in motility and chemotaxis. The microarray data

confirmed previous studies about the influence of temperature on motility (2, 201), and could be easily confirmed by motility tests. In *E. coli*, the flagellar operons are divided into three classes with regard to their relative position in the transcriptional hierarchy (200, 268, 310, 322). According to the transcriptome comparison of the two strains, the class 2 and 3 genes were downregulated at 41 °C relative to 37 °C. This was confirmed by real-time RT-PCR for one representative gene of each class. Genes which are involved in flagellum biogenesis (for reviews of flagellar hierarchy, see references (7, 58, 179, 322)) at different levels, were investigated by real-time RT-PCR i.e. the chemotaxis genes *cheA*, *cheZ motA* (class III genes), the *flhD* (classI), the *fliA* (class II) gene, *fliC* (class III), the *aer* and *tsr* gene (class III). These confirmed the microarrays data and phenotypic assays.

In case of class I, which only comprises the genes *flhC* and *flhD*, reduced transcription levels at 41 °C could not be detected by microarray hybridization and real-time PCR. However, it has been demonstrated that the regulation of the FlhDC complex can also occur at the post-transcriptional level by the carbon storage regulator CsrA (350). Furthermore, post-translational regulation of FlhDC expression is mediated by the protease ClpX/ClpP in *Salmonella enterica* (325, 326). These regulatory mechanisms could also contribute to the repression of motility at higher temperature in strains IHE3034 and BEN374. In addition of the flagellar class II and class III genes, genes involved in chemotaxis were down regulated, too. This was also confirmed for *cheY* and *cheZ* by real-time RT-PCR.

Other de-regulated genes presented in the Tab. 22 and 23 (see Appendix) have an unknown or putative function. Interestingly, one of these genes, *yhjH*, was downregulated and has been recently characterized to have a function in motility (109). Based on sequence homology, *yhjH* belongs to the newly identified and highly prevalent family of proteins that function in the turnover of bis-(3’-5’)-cyclic dimeric guanosine monophosphate (c-di-GMP), which is a secondary messenger involved in the regulation of a wide variety of bacterial behaviors (283). The list of de-regulated genes may consist of further genes with a potential role in motility, but their potential function in signaling,

chemotaxis and signal transduction will have to be checked in the future. This may rapidly and easily provide new genes involved in motility. In Tab. 22 and 23 (see Appendix), genes like *yhjB*, *yihW* and *ynbD* may be good putative candidates for components of the sensory and signaling pathway that regulates motility. Based on BLAST analysis, *yihW* codes for a predicted DNA-binding transcriptional regulator, whereas *yhjB* and *ynbD* encode a predicted DNA-binding regulator of a two-component regulatory system and a predicted inner membrane phosphatase, respectively.

During a urinary tract infection, the role of motility is well known. Flagellum-driven motility allows bacteria to disseminate to sites more advantageous for colonization. The motility contributes to virulence, enabling UPEC to propagate in the bladder, to disseminate to the upper urinary tract (185) and establish pyelonephritis. This can then in certain cases develop into septicemia, a systemic propagation in the host. Therefore, it was unexpected to see the reduction of motility already at 41 °C. Previous experiments of this type have been done at 45 °C or higher. It is generally assumed that an infection is like a race between the colonizing pathogen and the host response. In this case, reduced motility may delay infection, and normally, should increase the probability of bacterial clearance by the host immune response. On the other side, the presence of less flagellar protein will reduce immune response against bacteria. Despite it, the virulence of the APEC strain is quite important, but the way of infection, is different, not only one way and may occur before birth. This, in addition of the host difference, may explain why the reduction of motility due to the temperature, has not a high incidence. In view of the data, we could not identify a specific regulator involved in the control of the motility by a temperature-dependent way. The hypothesis would be the existence or expression of a regulatory factor, known or unknown. The motility and its regulation are quite important in the virulence properties of pathogenic strains, and this motility effect *in vitro* is not an indicator of the motility *in vivo*, and explains why these strains are virulent.

Finally, the different studies on avian infection caused by *E. coli* indicate that the most important trait of APEC that contributes to avian colonization and development of

colibacillosis seems to be the serum resistance and resistance of APEC against macrophages (81, 93, 216, 260, 315). Nevertheless this motility effect must be analyzed in the future, or the ability of the strain to switch from a motile form to a less motile one.

6.1.4 Implications and outlook

In addition to the assessment of the general genome content of APEC and human ExPEC strains, we analyzed the transcriptomes of a human and avian O18:K1 isolate to characterize individual differences in virulence gene expression in response to their hosts, i.e. the different body temperatures. The results obtained for the two strains did not elucidate major differences between the human and avian isolate regarding regulation of their gene expression at the transcriptional level in response to human or avian body temperature. They rather mirror strain-specific effects and support a common behavior of the two strains even upon variation of one factor, i.e. the growth temperature of 37 °C or 41 °C. The body temperature is not the only difference between human and birds, and one must suspect a completely different gene expression pattern during infection, also in response to the course of infection, e.g. early and late stage of infection. The results obtained from the transcriptome, genome as well as phenotypic comparison of human ExPEC and APEC, further supports earlier studies (98, 99, 167, 222, 285) which discuss that APEC and certain human ExPEC may be zoonotic. APEC are at least considered a reservoir for virulence-associated genes of human ExPEC and comprise strains with a high pathogenic potential for humans (99). The transmission between humans and birds has is not been properly investigated until now. Further studies on transcriptional and protein level should be performed to evaluate this zoonotic risk for humans due to the economic and hygienic impact on our society. This is the first study which unravels considerable overlaps between human and animal ExPEC strains as well as putative host-specific responses at the transcriptional level. It becomes more and more apparent that the presence of a common set of virulence-associated genes among ExPEC strains as well as similar gene expression patterns and phylogenetic backgrounds indicate a significant zoonotic risk of avian-derived *E. coli* isolates.

6.2 Characterization of Mat fimbriae expression of *E. coli* strain IHE3034

Bacterial adherence to host tissues is a complex process that, in many cases, involves the participation of several distinct adhesins, which may act at the same time or at different stages during infection. These fimbriae are more represented among pathogenic strains than among non-pathogenic strains (75.45% vs 55.5% (83), 100% vs 40% (362), 90.4% vs 26.7 (214)) and have been longtime considered as potential virulence factors of ExPEC. These fimbriae are factors contributing to the virulence potential of such strains, but they are not necessarily sufficient to cause disease (219). This may be due to the large amount of unknown or uncharacterized adhesins and putative adhesins which may be atypical or not expressed under the studied conditions. The recently identified Mat (meningitis associated and temperature regulated) fimbriae, named because the expression of this fimbrial type was found to be associated with the major clonal group of MENEC, O18:K1:H7, as well as with low temperature (262), is one of these new putative virulence-associated adhesins. In the last years, a few studies have been done on these fimbriae despite the fact the gene cluster is very well conserved in the majority of *E. coli*.

Whereas Poutu *et al.* reported that the expression of these fimbriae was specific for the serotype O18:K1 and K2 as well as temperature-dependant, Rendon and colleagues (276) demonstrated in EHEC that these fimbriae were well expressed in a lot of intestinal pathogenic *E. coli* strains at 37 °C in DMEM cell culture medium with 5 % CO_2 concentration. Our own attempts to express Mat fimbriae of O18:K1 and K2 strains under these conditions in RPMI cell culture medium have not been successful. Rendon and co-workers designated these fimbriae "*E. coli* common pilus" (ECP), which are composed of a 21-kDa pilin subunit whose amino acid sequence corresponds to the product of the gene *yagZ* (designated *matB* by Poutu *et al.*, and *ecpA* by Rendon *et al.*). ECP production was demonstrated in 71.6 % of a total of 169 *ecpA*+ strains representing intestinal and extraintestinal pathogenic (121 of a total of 169 strains) as well as commensal fecal *E. coli* isolates (16). They estimated that *in vivo* all the *ecpA*-positive strains that were phenotypically negative should be able to express Mat fimbriae, but

depending on the niche and other growth conditions. Furthermore, Mat fimbriae-mediated adhesion to cultured epithelial cells could be shown, but nothing else was known about the expression and function of this new fimbrial type. The organization of the gene cluster has only been investigated to a certain extent by *in silico* analysis (262). The fact that in newborn meningitis isolates such as O18:K1:H7 strain IHE3034 Mat fimbriae are not expressed under the conditions described by Redon *et al.* implies that regulation of their expression is more complex and is a multifactorial process which needs to be further characterized. In this study, the role of the *matA* gene, coding for the putative regulator of Mat fimbriae has been characterized and its role for Mat fimbriae expression was analyzed. In addition, the predominance of this gene cluster in *E. coli* and the permanent expression of *matA* (with or without Mat fimbriae expression) let hypothesize a more general role for MatA than solely the control of Mat fimbriae expression.

6.2.1 Transcriptional organization and *matA* promoters differences between IHE3034 and MG1655

6.2.1.1 Genetic organization in two operons of *mat* gene cluster

To better understand the regulation of Mat fimbriae, the transcriptional organization of the gene cluster was analyzed. Poutu *et al.* already demonstrated cotranscription of *matB* and *matC* (262). Based on the results obtained by RT-PCR, we defined two operons: *matABCD* and *matEF* (Fig. 25). A bioinformatics analysis didn't reveal significant transcription start points for *matB* in the *matA*-*matB* intergenic region or within *matA*. Also based on the results of the determination of the *matB* transcriptional start point and the fact that primer extension indicated the transcriptional start site upstream of *matA*, the RT-PCR experiments have also been reproduced and clearly proved co-transcription of *matABCD* and *matEF*. These experiments further supported that the *matABCD* transcript is highly unstable, especially in the 5' and 3' end of *matA*, and let presume the effect is strongest at 37 °C than 20 °C. The smear detected for the *matA* transcripts by Northern blot analysis confirmed this, too.

6.2.1.2 *matA* promoters differences

In addition to the genetic organization of the *mat* gene cluster, the different regulation of mat expression has been analyzed in newborn meningitis isolates IHE3034 and K-12 strain MG1655. Although *E. coli* MG1655 carries the complete gene cluster, Mat fimbriae are not expressed. The previous work of Poutu *et al.* showed that IHE3034 lost expression of Mat fimbria at 20 ºC when the *matA* was deleted. On the other hand overexpression of MatA *in trans* was not sufficient to cause high level Mat expression in wt IHE3034 at 37 ºC or detectable expression in wt MG1655 strain at 20 °C or 37 ºC (Timo Lehti personal communication; and my own data).

The analysis and comparison of such fimbriae may have a good phylogenetic interest. Poutu *et al.*, Rendon *et al.* demonstrated that almost all the *Escherichia coli* strains present the *mat* cluster, completely or not. They performed a PCR-based *matB* survey in a collection of 176 strains representing NFEC (normal flora *E.coli*) and the major *E. coli* pathogroups (EHEC, enteropathogenic, enterotoxigenic, enteroaggregative, enteroinvasive, rabbit pathogenic, avian pathogenic, and uropathogenic). This gene was present in 169 (96%) of these strains. The last 4% missed the *matA*, *matB*, *matC* and *matD* genes, without any data for *matE* and *matF*. Differences in the *matA* and *matB* nucleotide sequences and also in their deduced amino acid sequences could be detected between strains IHE3034 and MG1655 (Fig. 29 and 30). Unfortunately, in the absence of a known target of the MatB protein, it is difficult to analyze the effect of these variations on the MatB secondary structure which may affect this protein's receptor specificity. Additionally, the mutations observed in MatA could not be correlated with a different Mat fimbriae expression without purified MatA protein. Consequently, purification of heterologously expressed MatA by affinity chromatography resulted in a co-purification with the chaperone GroEL and no DNA binding capacity of MatA (Timo Lehti, personal communication). For this reason, the focus was set in this thesis on further *in silico* analyses. These analyses predicted different putative promoters and transcription factor binding sites in strains IHE3034 and MG1655 (Fig. 23). To validate the *in silico*

predictions, the exact transcriptional start of *matA* was determined by 5'-RACE analysis. In the two *E. coli* strains, three transcription start points were identified. Two of them were common for both strains, but one specific transcriptional start could be detected in each of them (Fig. 26). These data suggest that individual transcriptional start sites may contribute to the differences in Mat fimbriae expression between both strains. Furthermore, these results demonstrate that *matA* may be expressed, but not Mat fimbriae. In addition to MatA, other transcription factors affect Mat fimbriae expression. We hypothesize that the different transcriptional start points of *matA* influence mRNA stability. The detection of *matA* transcripts by Northern blot analysis as well as the results of the 5'-RACE analysis support the idea that, at least, the 5'-end of the *matA* transcript is very unstable. For each strain, the most distal promoter could not well be determined whereas the other two transcriptional starts were clearly identified.

The *matA* gene with its own promoter could not be easily amplified from strain IHE3034 by PCR. This may be indicative of marked DNA curvature of this promoter region. Pronounced DNA curvature at promoter regions is common and often involved in regulation of transcription (79, 213). In contrast to strain IHE3034, *matA* with its promoter could be easily amplified in strain MG1655. Analysis of electrophoretic mobility of DNA fragments detected a region with marked DNA curvature at potential *matA* regulatory regions in strain IHE3034, but not in *E. coli* MG1655 (Timo Lehti, personal communication). In addition, the *mat* upstream region in *E. coli* IHE3034 lacks one of two potential GATC methylation sites which are present in K-12 strain MG1655. The methylation state of regulatory DNA regions has been shown to influence H-NS dependent P fimbriae expression (356).

In summary, these analyses indicate several variations in DNA sequence and DNA curvature that may be involved in differential regulation of Mat fimbriae expression *in vitro* and/or *in vivo* as well as between *E. coli* strains IHE3034 and MG1655 (see Figure 36).

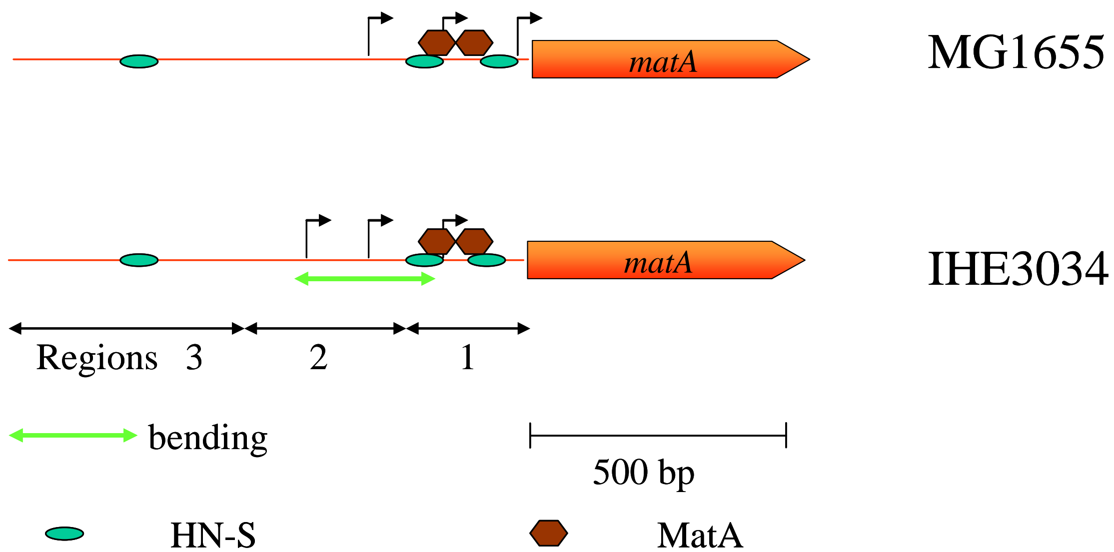


Figure 36: Summary of differences and similarities between the *matA* promoter structure and organization in IHE3034 and MG1655. Another difference, not indicated on the figure, is the presence of one potential GATC methylation sites in IHE3034 compared to K-12 strain MG1655.

The exchange of the *matA* promoter between both strains would further support the *in silico* findings. Based on the *in silico* data and on our results, the *matA* upstream region was subdivided in three parts and these regions were exchanged between *E. coli* strains IHE3034 and MG1655. Mat fimbriae expression was detectable in *E. coli* MG1655 when the regions 1 and 2 were exchanged by those of strain IHE3034 and, in analogy, their expression was completely abolished in corresponding variant of *E. coli* IHE3034. Consequently, the region 3 was not involved in regulation of Mat fimbriae expression. In addition, the regions 1 and 2, which contain the majority of sequence differences (promoters, transcription factor binding sites) between strains IHE3034 and MG1655, have thus been shown to be essential for Mat fimbriae expression. Interestingly, exchange of region 1 and 2, which comprises the DNA region with marked DNA curvature only in the human ExPEC, between strains IHE3034 and MG1655 did not result in the same expression level of Mat fimbriae in strain MG1655 as in *E. coli* IHE3034 (Timo Lehti, personal communication). This may be due, at least partially, to the different MatA amino acid sequences in both strains which may have co-evolved with its own promoter. Consequently, the MatA protein from *E. coli* MG1655 may bind to the *matA* promoter of strain IHE3034 with lower efficiency than to its own promoter.

Whether some of these factors are responsible for the differential expression or whether these factors act in a co-ordinate manner needs to be elucidated in future studies.

To perform this study, it was required to work with purified and functional MatA protein alone, without the chaperon GroEL. Functional MatA protein could be purified using a fusion protein of the maltose binding protein and MatA (MBP-MatA, this study). With this, binding of MatA to DNA could be demonstrated for the first time (Timo Lehti, personal communication) and its binding could be localized within the *matA* promoter region (between -10 to 608 nt) in *E. coli* IHE3034 and MG1655 (Timo Lehti, personal communication).

6.2.1.3 H-NS affects the Mat fimbriae expression

Another aspect of regulation of Mat fimbriae expression was the investigation of the impact of the histone-like protein H-NS. This global regulator affects the expression of a high amount of genes (more than 5% in *E. coli*) (15, 146), as it binds to DNA thus changing it’s curvature (67, 68). In addition, other H-NS-like proteins were recently identified to be involved in virulence gene regulation (213, 330, 354, 357).

In addition, DNA binding assays with H-NS and the same *matA* upstream region demonstrated that H-NS bound with different affinity to the DNA stretches from *E. coli* IHE3034 and MG1655 (Timo Lehti, personal communication). Comparison of H-NS binding to *mat* regions showed that the IHE3034-specific DNA fragment located -754 to -1342 bp upstream of the *matA* translational start had a higher affinity for H-NS than the corresponding region from strain MG1655. In summary, three differences with respect to the DNA sequence, the presence of transcriptional start and transcription factor binding sites as well as the structure of the *matA* upstream region have been identified in *E. coli* IHE3034 and MG1655. These differences may potentially contribute to the differential expression of Mat fimbriae in these strains. Whether some of these factors are sufficient to cause differential expression or whether these factors function in concert will have to be elucidated in future studies.

H-NS-dependent repression of gene expression is often induced by environmental signals through the action of transcriptional activators which alleviate H-NS dependent repression (15). Interestingly, a different role of H-NS for Mat fimbriae expression in response to the growth temperature could be observed. Mat fimbriae expression of strain IHE3034 was studied in the wild type, as well as in the isogenic *hns*-, or *hns matA* mutants in whole cell ELISA assays at different temperatures (Timo Lehti, personal communication). At 20 ºC, the highest Mat fimbriae expression was observed in *E. coli* IHE3034 *hns* in the presence and absence of *matA*. In contrast, at 37 ºC the presence of *matA* was required for Mat expression in the *hns*-negative background. The IHE3034 *hns matA* double mutant expressed Mat fimbriae at 20 ºC, but not at 37 ºC because MatA is required in the *hns*-negative background at 37 °C for Mat expression. Obviously, Mat fimbriae expression is also regulated by a MatA and H-NS-independent temperature-sensitive mechanism which favours fimbriae production at low temperatures. It can be concluded that at 20 ºC MatA functions as an H-NS antagonist and at 37 ºC it counteracts repression by an unknown mechanism. H-NS represses gene expression by binding upstream and/or downstream of promoter regions hence hindering RNA polymerase function (15, 354, 359). Remarkably, temperature (20 or 37 ºC) did not affect the affinity of H-NS to the *matA* coding region and hence the lack of Mat expression in strains bearing an intact *matA* region is probably not due to H-NS binding to this region (Timo Lehti, personal communication).

6.2.2 Biological function of *matA* and Mat fimbriae

6.2.2.1 The role of MatA on *matABCD* transcription

To asses the role of a regulator and globally screen for its potential targets in an organism, transcriptome analysis is one of the best tools to screen all the genes of an organism if the genome sequence is avalaible. In this study, the global transcriptome of IHE3034 wild type and its isogenic *matA* mutant has been compared at 20 °C because this is one of the conditions under which Mat fimbriae are expressed directly under the

influence of MatA. The microarray data obtained and the subsequent confirmation of the most relevant results by real-time RT-PCR confirmed the role of MatA on *matABCD* transcription, but not on transcription of *matEF*. The negative role of H-NS on Mat fimbriae expression was also proven at 20 °C and 37 °C, for the complete *mat* gene cluster. A stronger effect was observed for the *matABCD* operon than for *matEF*. Any other gene or operon was significantly affected in the absence of MatA under the chosen experimental conditions.

Nevertheless, after the construction of the MBP-MatA fusion protein, it was demonstrated that the isogenic isogenic *matA* mutant produce a MatA protein still able to bind DNA, despite the absence of Mat fimbriae expression. In this case, few side effects must still be present and may explain the low amount of deregulated genes, especially the lack of genes involved in motility.

6.2.2.2 Effect on biofilm formation and on the motility

The *matA* mutant was also phenotypically compared with the wild type strain to characterize possible differences in the expression of virulence-associated traits. Differences in expression of the toxin α-hemolysin, fimbrial adhesins, and iron acquisition systems, could not be found. Interestingly, *matA* inactivation drastically affected biofilm formation of *E. coli* IHE3034 (Fig. 31): strong biofilm formation was observed under the same conditions under which Mat fimbriae were expressed. Biofilm formation was reduced when Mat fimbriae were not expressed. Complementation of the *matA* or *matBCDEF* mutants indeed correlated the different biofilm formation with Mat fimbriae expression (Fig. 32). This observation confirmed a new function of these fimbriae. Biofilms are considered a virulence-associated trait. Biofilm formation of *Enterobacteriaceae* is believed to play a significant role for the colonization and establishment of infections on mucosal surfaces and may also enhance microbial survival in the environment (37, 63). Mat fimbriae-dependent biofilms may be formed in the environment as well as in the host, because Rendon and coworkers reported on Mat fimbriae expression in cell culture and their role for adhesion to eukaryotic cells. This

supports the virulence-associated role of these fimbriae, and due to its expression at room temperature, maybe a factor of nosocomial infection, too. They may represent a good alternative or complementary factor involved in *E. coli* biofilm formation and may be a good target to interfere with biofilm formation. The preponderant role of this fimbrial type in the biofilm formation at low temperature opens up new perspectives in the comprehension of biofilm formation and the role of Mat fimbriae for host colonization, especially as Mat fimbriae are expressed in cell culture at 37 °C.

The most unexpected result of the phenotypic comparison of wild type strains MG1655, BEN374 and IHE3034 and their mutants deficient in Mat fimbriae expression was the increased motility of the latter ones (Fig. 33 and 34). According to the transcriptome data obtained with strain IHE3034 *matA*H179P, de-regulation of genes known to be involved in motility and chemotaxis was not observed upon *matA* inactivation. The microarray data were also confirmed by real-time RT-PCR.

Nevertheless, the construction of the MBP-MatA protein fusion and its isogenic mutant MBP-MatAH179P demonstrated the possibility of MatA to bind *matA* promoter, despite the lack of Mat fimbriae expression in this strain (Timo Lehti, personal communication). In this case, side effects must be present, explaining the weak amount of genes deregulated. It clarified too the strongest effects on motility test in IHE3034 *ΔmatA* (data not show) and its confirmation on Western blot in this mutant vs. wt (see Figure 45, appendix). In view of all these data, the reproducibility of this effect in others strains (MG1655, BEN374), and linked to the fact that the absence of either a functional *matA* gene or that of the structural genes *matBCDEF* independently resulted in increased motility in different strain backgrounds indicates that this is indeed a general regulatory cross-talk that most likely occurs directly or indirectly on the level of translation or stability of components of the flagella apparatus or other factors that affect flagellation or chemotaxis. The major regulator component of the flagella apparatus, *flhDC*, and especially its promoter must be investigated in presence of MBP-MatA.

The motility of bacteria implements a complex regulatory network which involves different stimuli and genes that affect flagellation (268). Sjostrom *et al.* demonstrated that another protein encoded in a fimbrial gene cluster also affects motility and type 1-fimbriae (300). There seems to be a frequent cross-talk between fimbriae and flagella. This effect is present, too, in case of Mat fimbriae.

The impact of Mat fimbriae in virulence was tested by infection of chickens. APEC strain BEN374 was used to infect chickens by inoculation of the airsac. In addition to the wild type strain, two mutants have been used for infection as well: one was deleted for *matA* and the other for *matB*. Virulence of both mutants which does not express Mat fimbriae was, however, not significantly reduced relative to the wild type (P. Germon, Nouzilly, data not shown). Due to the inappropriate mutation, and the genomic instability of the strain IHE3034 (data not show), new animal experiment must be performed in an appropriate model and and with a more relevant strain.

6.2.3 Implications and outlooks

The results obtained in this thesis will form the basis for further experiments aiming at the detailed characterization of the *mat* gene cluster, regulation of Mat fimbriae expression and to decipher the specific role of MatA, which has a constitutive expression (see figure 37).

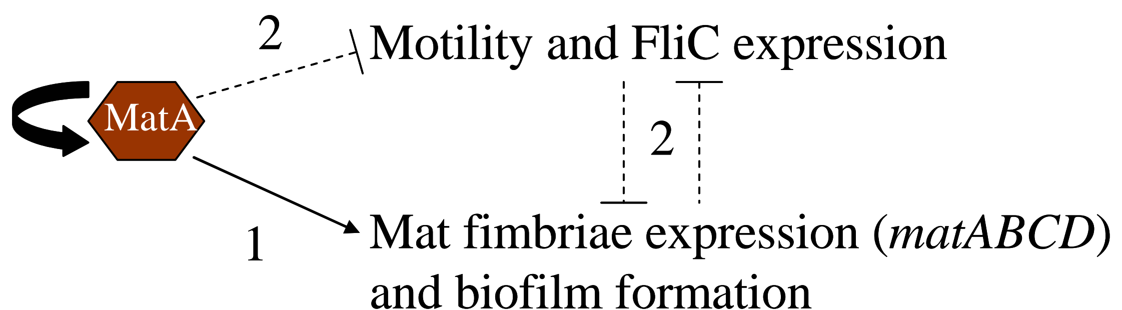


Figure 37: Summary on the MatA regulation functions. 1) Direct regulation in case of Mat fimbriae expression; 2) for the motility and FliC expression, it needs to be further analyzed if it is due to a cross-talk between fimbriae and flagella, or a direct regulation by MatA.

To increase the knowledge on the role of MatA, this protein should be purified and its DNA binding motif should be determined. The functional characterization of Mat fimbriae should focus on the cellular receptor on eukaryotes or in the extracellular matrix and the regulation of their expression *in vivo* in different isolates. To analysis of the impact of MatA on motility may involve the characterization of an unknown regulatory mechanism that affects expression of the motility genes.

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8. Appendix

8.1 Nucleotide and deduced amino-acid sequence of the N-terminal part of *fimH* in the colirisk strains

fimH K12

ATGAAACGAGTTATTACCCTGTTTGCTGTACTGCTGATGGGCTGGTCGGTAAATGCCTGGTCATTCGCCT
GTAAAACCGCCAATGGTACCGCTATCCCTATTGGCGGTGGCAGCGCCAATGTTTATGTAAACCTTGCGCC
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fimH RS218

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fimH IHE3034

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fimH BEN79

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fimH BEN374

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fimH BEN2908

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fimH IHE3072

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fimH 1772

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fimH AC/I ou 789

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fimH 285

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8.2 Nucleotide and deduced amino-acid sequence of the N-terminal part of *matA* in the colirisk strains

> *matA* seq in K12

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>*matA* seq in IHE3034

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> *matA* BEN374

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>*matA* 1772

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>*matA* 285

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>*matA* BEN2932 (AC/I)

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GATTAATTTATCTATCGTTGGAGAAAATTAGAAAAGATTTCGTGTTTATCAATCTCAGTACGGATTCTTTA
TCTGAATTTATAAACCGTGATAATGAATGGTTATCCGCGGTAAAGGGGAAACAGGTCGTATTGATTGCGGC
CAGAAAGTCAGAAGCCTTAGCAAATTATTGGTATTACAATAGCAATATTAGGGGCGTGGTATACGCTGGAC
TGAGTCGTGATATTAGAAAAGAACTGGCCTATGTGATTAATGGCAGGTTCCTGAGAAAAGATATTAAGAAA
GATAAAATCACGGACCGGGAAATGGAAATTATCCGCATGACGGCCCAGGGAATGCAACCTAAATCGATTGC
CAGAATTGAAAATTGTAGTGTGAAGACAGTGTATACCCATCGGCGTAATGCTGAGGCCAAGCTGTACTCAA
AAATATATAAGTTGGTTCAGTAA

MTWQSDYSRDYEVKNHMECQNRSDKYIWSPHDAYFYKGLSELIVDIDRLIYLSLEKIRKDFVFINLSTDSL
SEFINRDNEWLSAVKGKQVVLIAARKSEALANYWYYNSNIRGVVYAGLSRDIRKELAYVINGRFLRKDIKK
DKITDREMEIIRMTAQGMQPKSIARIENCSVKTVYTHRRNAEAKLYSKIYKLVQ

>*matA* IHE3072

GTGACATGGCAAAATGATTACAGCAGGGACTATGAGGTTAAAAACCATATGGAATGTCAAAACCGTTCTGA
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ACTGAGTTTATAAACCGTGATAATGAGTGGTTATCCGCGGTAAAGGGGAAACAGGTCGTATTGATTGCGGC
CAGAAAGTCAGAAGCCTTAGCAAATTATTGGTATTACAACAGCAATATTAGGGGCGTGGTATACGCTGGAC
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CAGAATTGAAAATTGTAGTGTGAAGACAGTGTATACCCATCGGCGGAATGCAGAGGCCAAGCTGTACTCAA
AATTATATAAGTTGGTTCAGTAA

MTWQNDYSRDYEVKNHMECQNRSDKYIWSPHDAYFYKGLSELIVDIDRLIYLSLEKIRKDFVFINLNTDSL
TEFINRDNEWLSAVKGKQVVLIAARKSEALANYWYYNSNIRGVVYAGLSRDIRKELAYVINGRFLRKDIKK
DKITDREMEIIRMTAQGMLPKSIARIENCSVKTVYTHRRNAEAKLYSKLYKLVQ

>*matA* RS218

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GATTAATTTATCTATCGCTGGAGAAAATCAGAAAAGATTTCGTGTTTATCAATCTCAATACGGATTCTTTA
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GATAAAATCACTGACCGGGAAATGGAAATTATCCGCATGACGGCTCAGGGAATGCTGCCTAAATCGATTGC
CAGAATTGAAAATTGTAGTGTGAAGACAGTGTATACCCATCGGCGGAATGCAGAGGCCAAGCTGTACTCAA
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MTWQNDYSRDYEVKNHMECQNRSDKYIWSPHDAYFYKGLSELIVDIDRLIYLSLEKIRKDFVFINLNTDSL
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DKITDREMEIIRMTAQGMLPKSIARIENCSVKTVYTHRRNAEAKLYSKLYKLVQ

8.3 Nucleotide and deduced amino-acid sequence of the N-terminal part of *matB* in the colirisk strains

>*matB* seq in K12

ATGAAAAAAAAAGGTTCTGGCAATAGCTCTGGTAACGGTGTTTACCGGCATGGGTGTGGCGCAGGCTGCTGA
CGTAACAGCTCAGGCTGTAGCGACCTGGTCGGCAACAGCCAAAAAAGACACCACCAGTAAGCTGGTTGTGA
CGCCACTCGGTAGCCTGGCGTTCCAGTATGCCGAAGGCATTAAAGGTTTTAACTCACAGAAAGGTCTATTT
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AACCCAGTTGGATACCTCAGGTTCCACACTGAATGTGGGCGTGGATTATAACGGCGCGGCAGTCGAAAAAA
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TACAATGCCAGCAATCGTACCACCGCACAGGATGGTTTCACCTTCTCCATCATCAGCGGTACCACCAATGG
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ACGCGACCTGGACCAGTTAA

MKKKVLAIALVTVFTGMGVAQAADVTAQAVATWSATAKKDTTSKLVVTPLGSLAFQYAEGIKGFNSQKGLF
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YNASNRTTAQDGFTFSIISGTTNGTTAVTDYSTLPEGIWSGDVSVQFDATWTS*

>*matB* IHE3034

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AACCCAGTTGGATACCTCAGGTTCCACACTGAATGTGGGCGTGGATTATAACGGCGCGGCAGTCGAAAAAA
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TACAATGCCAGCAATCGTACCACCGCACAGGATGGTTTCACTTTCTCCATCATCAGCGGCACCACCAATGG
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>*matB* BEN374

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AACCCAGTTGGATACCTCAGGTTCCACACTGAATGTGGGCGTGGATTATAACGGCGCGACAGTCGAAAAAA
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TACAATGCCAGCAATCGTACCACCGCACAGGATGGTTTCACTTTCTCCATCATCAGCGGCACCACCAATGG
TACCACCGCAGTAACCGATTACAGCACTCTACCGGAAGGCATCTGGAGCGGCGACGTTAGCGTACAGTTCG
ACGCTACCTGGACCAGTTAA

MKKKVLAIALVTVFTGTGVAQAADVTAQAVATWSATAKKDTTRKLVVTPLGSLAFQYAEGIKGFNSQKGLF
DVAIEGDSTATAFKLTSRLITNTLTQLDTSGSTLNVGVDYNGATVEKTGDTVMIDTANGVLGGNLSPLANG
YNASNRTTAQDGFTFSIISGTTNGTTAVTDYSTLPEGIWSGDVSVQFDATWTS*

>*matB* 1772

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>*matB* BEN2908

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>*matB* BEN79

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AACCCAGTTGGATACCTCAGGTTCCACACTGAATGTGGGCGTGGATTATAACGGCGCGACAGTCGAAAAAA
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TACAATGCCAGCAATCGTACCACCGCACAGGATGGTTTCACTTTCTCCATCATCAGCGGCACCACCAATGG
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MKKKVLAIALVTVFTGTGVAQAADVTAQAVATWSATAKKDTTSKLVVTPLGSLAFQYAEGIKGFNSQKGLF
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>*matB* 285
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AACCCAGTTGGATACCTCAGGTTCCACACTGAATGTGGGCGTGGATTATAACGGCGCGGCAGTCGAAAAAA
CTGGCGATACCGTGATGATCGATACCGCCAACGGCGTACTGGGCGGCAACCTTAGCCCGCTGGCTAACGGT
TACAATGCCAGCAATCGTACCACCGCACAGGATGGTTTCACCTTCACCATCATCAGCGGCACCACCAATGG
TACCACCGCAGTAACCGATTACAGCACTCTACCGGAAGGCATCTGGAGCGGCGACGTTAGCGTACAGTTCG
ACGCGACCTGGACCAGTTAA

MKKKVLAIALVTVFTGMGVAQAADVTAQAVATWSATAKKDTTSKLVVTPLGSLAFQYAEGIKGFNSQKGLF
DVAIEGDSTATAFKLTSRLITNTLTQLDTSGSTLNVGVDYNGAAVEKTGDTVMIDTANGVLGGNLSPLANG
YNASNRTTAQDGFTFTIISGTTNGTTAVTDYSTLPEGIWSGDVSVQFDATWTS*

>*matB* BEN2932 (AC/I)
ATGAAAAAAAAAGGTTCTGGCAATAGCTCTGGTAACGGTGTTTACCGGCATGGGTGTGGCGCAGGCTGCTGA
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>*matB* IHE3072
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TACAATGCCAGCAATCGTACCACCGCACAGGATGGTTTCACTTTCTCCATCATCAGCGGCACCACCAATGG
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MKKKVLAIALVTVFTGTGVAQAADVTAQAVATWSATAKKDTTSKLVVTPLGSLAFQYAEGIKGFNSQKGLF
DVAIEGDSTATAFKLTSRLITNTLTQLDTSGSTLNVGVDYNGAAVEKTGDTVMIDTANGVLGGNLSPLANG
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>*matB* RS218
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GACGTGGCTATCGAGGGTGACTCAACGGCTACCGCCTTTAAACTGACCTCACGCCTTATCACCAACACCTT

AACCCAGTTGGATACCTCAGGTTCCACACTGAATGTGGGCGTGGATTATAACGGCGCGACAGTCGAAAAAA
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TACAATGCCAGCAATCGTACCACCGCACAGGATGGTTTCACTTTCTCCATCATCAGCGGCACCACCAATGG
TACCACCGCAGTAACCGATTACAGCACTCTACCGGAAGGCATCTGGAGCGGCGACGTTAGCGTACAGTTCG
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MKKKVLAIALVTVFTGTGVAQAADVTAQAVATWSATAKKDTTSKLVVTPLGSLAFQYAEGIKGFNSQKGLF
DVAIEGDSTATAFKLTSRLITNTLTQLDTSGSTLNVGVDYNGATVEKTGDTVMIDTANGVLGGNLSPLANG
YNASNRTTAQDGFTFSIISGTTNGTTAVTDYSTLPEGIWSGDVSVQFDATWTS*

8.4 *In silico* analysis of *matA* promoters by Bprom

The start codon of *matA* CDS is represented in capital letters.
(http://www.softberry.ru/berry.phtml?topic=bprom&group=programs&subgroup=gfindb)

In MG1665

```
gatttgtaaatctaatccacgttttaaggccgttcagggtcgtaagaaaaaacgttgatt
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```

```
> test sequence
 Length of sequence-      1491
 Threshold for promoters -  0.20
 Number of predicted promoters -       4
 Promoter Pos:   1000 LDF-   6.15
 -10 box at pos.    985 aattaaact Score    57
 -35 box at pos.    966 tttaag    Score    35
 Promoter Pos:    262 LDF-   4.27
 -10 box at pos.    247 ctgtatgct Score    57
 -35 box at pos.    223 ttgact    Score    61
 Promoter Pos:    656 LDF-   2.36
 -10 box at pos.    641 cattaaaat Score    68
 -35 box at pos.    623 ttgtgc    Score     8
 Promoter Pos:   1451 LDF-   1.93
 -10 box at pos.   1436 tggtatacg Score    46
 -35 box at pos.   1413 ttacaa    Score    32

 Oligonucleotides from known TF binding sites:

 For promoter at   1000:
     rpoD16:  AAATAATT at position     981 Score -   15
```

```
       argR:  AATAATTA at position     982 Score -  17
       arcA:  TAATTAAA at position     984 Score -  11
 For promoter at    262:
     rpoD16:  TCGCCCCT at position     241 Score -   7
 No such sites for promoter at    656
 No such sites for promoter at   1451
```

In IHE3034

```
gatttgtaaatctaatccacgttttaaggccgttcagggtcgtaagaaaaaacgttgatt
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cgctggactgagtcgtgatattagaaaagaactggcctatgtgattaatgg
```

```
> test sequence
 Length of sequence-      1491
 Threshold for promoters -  0.20
 Number of predicted promoters -      4
 Promoter Pos:    729 LDF-   7.81
 -10 box at pos.    714 ATTTAAACT Score    64
 -35 box at pos.    694 TTTATA    Score    39
 Promoter Pos:    260 LDF-   4.93
 -10 box at pos.    245 CTGTATGCT Score    57
 -35 box at pos.    222 TTGACT    Score    61
 Promoter Pos:   1038 LDF-   3.54
 -10 box at pos.   1023 ACGTATCTT Score    37
 -35 box at pos.   1001 TTAACC    Score    21
 Promoter Pos:   1449 LDF-   1.93
 -10 box at pos.   1434 tggtatacg Score    46
 -35 box at pos.   1411 ttacaa    Score    32

 Oligonucleotides from known TF binding sites:

 For promoter at    729:
        fis:  TCTTTAAT at position     670 Score -   6
       phoB:  TTTAATTA at position     672 Score -  11
     rpoD17:  TTATGATA at position     684 Score -   7
```

```
       fis:  TATTCTAT at position     698 Score -  10
      lexA:  TTTTTTTA at position     725 Score -  16
For promoter at    260:
    rpoS17:  TTATATTA at position     211 Score -  14
For promoter at   1038:
    rpoD16:  AAATAATT at position     978 Score -  15
      argR:  AATAATTA at position     979 Score -  17
      arcA:  TAATTAAA at position     981 Score -  11
No such sites for promoter at   1449
```

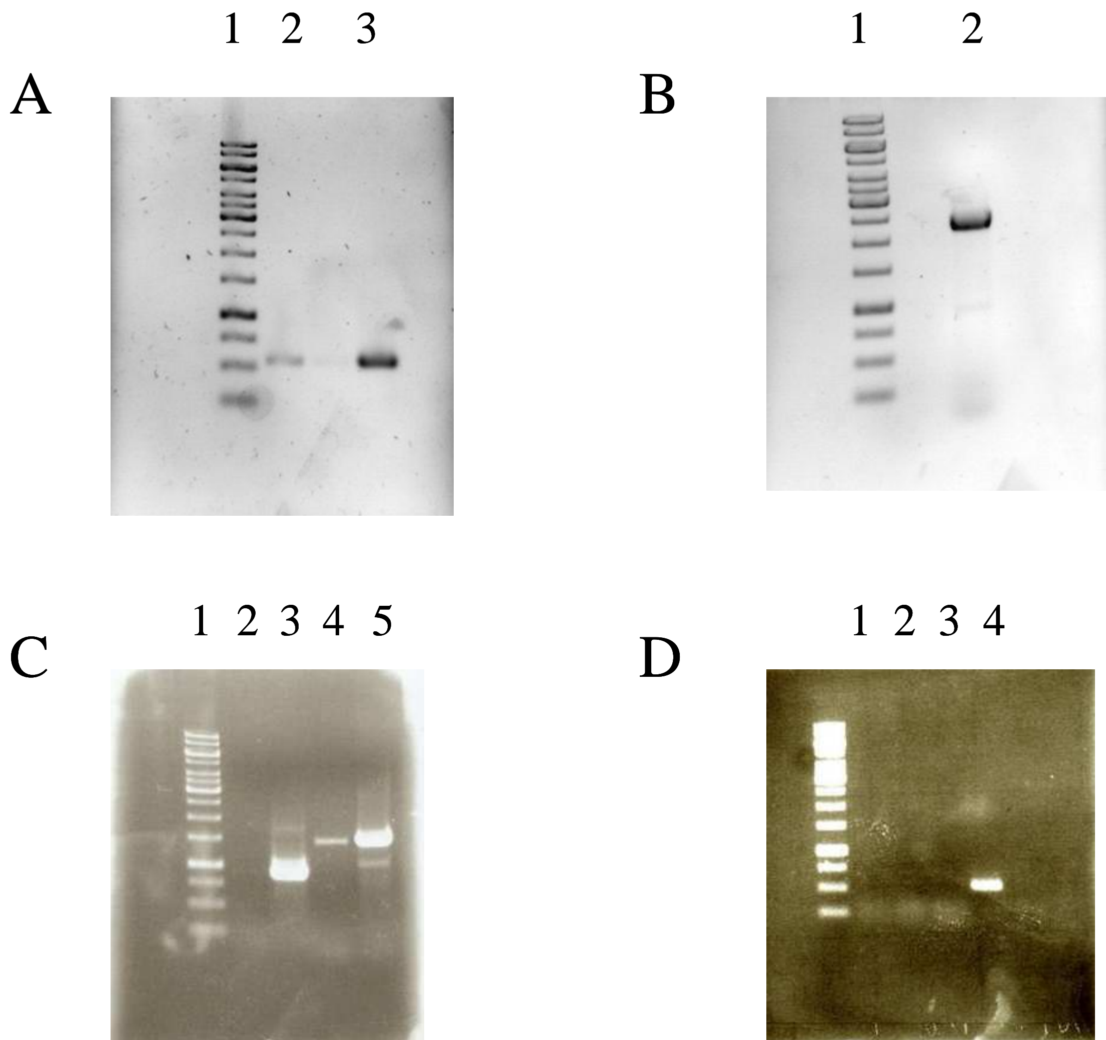

Figure 38: Trancriptional organization of the Mat fimbriae genes by RT-PCR. (A) Cotranscript between *matA* and *matB* 1: DNA ladder; 2: cDNA; 3: DNA. (B) Cotranscript between *matA* and *matD.* The size of the cotranscript fit with the theorical one, including the orf of *matB* and *matC*. 1: DNA ladder; 2: cDNA. (C) In the line 2, the amplification of a transcript product between *matD* and *matE* is negative. It isn't the case in the line 3 between *matE* and *matF*. 1: DNA ladder; 2: cDNA; 3: DNA; 4: cDNA; 5: DNA. (D) Control of the absence of DNA in the RNA samples used in these experiments. 1, 2, 3: RNA samples; 4: DNA sample (control).

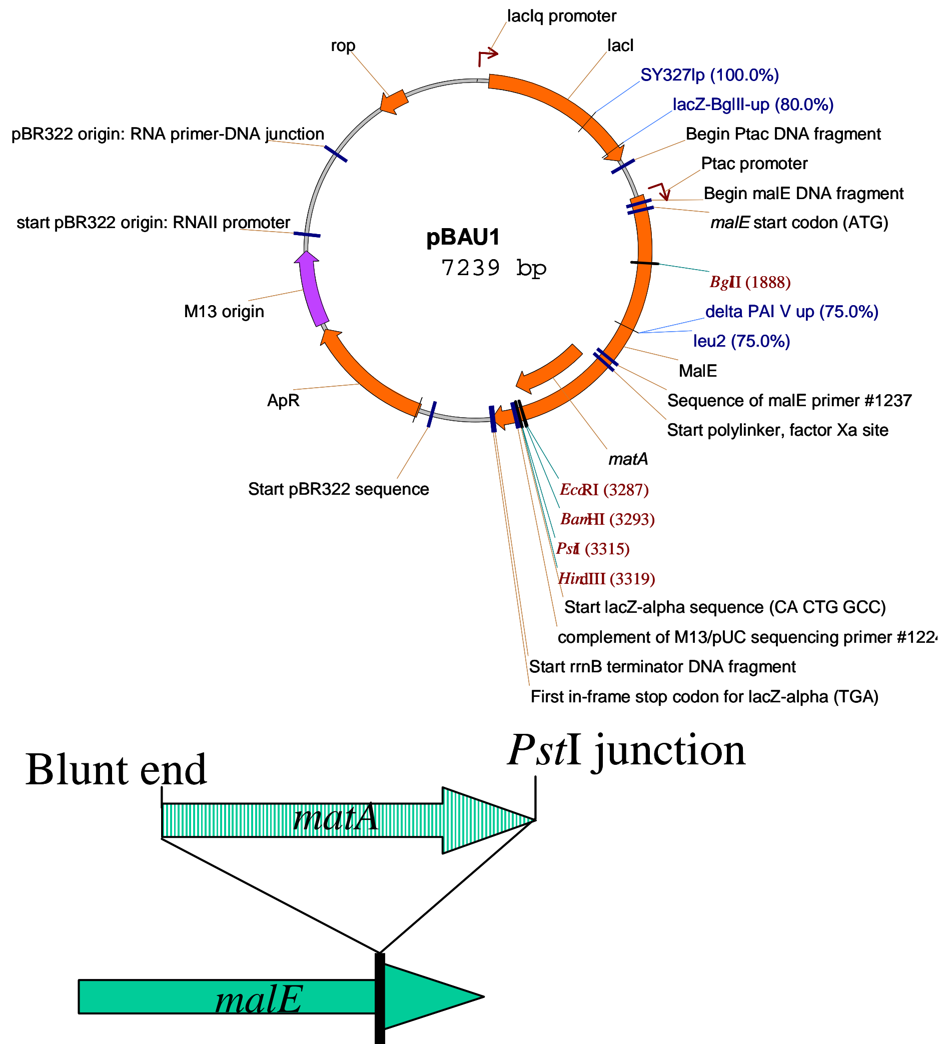

Figure 39: Map of plasmid pBAU constructed during this work. Plasmid pBAU1 was constructed by cloning a PCR-generated fragment encoding MatA from IHE3034. This fragment was a blunt-end and contains a *Pst*I site. It has been purified and digested by the restriction enzyme *Pst*I. pMAL-c2x was digested by *Xmn*I which produces a blunt end and by *Pst*I which does a « sticky » end. The primers 1224 and 1237 were used for sequencing of the plasmid.

Table 19: Significant up regulated genes in IHE3034 compared to *matA* mutant at 20 °C. p≤ 0.05
sd: standard deviation

	Mean (Stats)	SD (Stats)		
E100000285	3,697	0.333	*yagZ*	hypothetical protein
E100000286	2,188	0.484	*ykgK*	CDS
E100002824	2,603	0.019	*ECs3760*	-
E200000691	2,781	0.818	*ECs0323*	-
E200000690	3,433	1,038	*b0292*	*yagY*
E200000372	1,254	0.203	*b2390*	*ypeC*
E100002347	1,274	0.228	*ypeC*	CDS
E100001010	2,724	0.08	*b1037*	*csgG*
E100000284	3,197	0.664	*b0292*	*yagY*
E200000154	1,043	0.416	*b4060*	*yjcB*
E200002134	1,097	0.038	*c3386*	-
E200000024	1,335	0.058	*ECs0376*	-
E200001368	2,056	0.93	*b1309*	*ycjM*
E200001350	1,233	0.059	*ECs1835*	-
E100003952	1,233	0.562	*Z5659*	*yjcB*
E100004910	3,112	0.18	*Z1444*	-
E100000918	1,704	0.108	*b0943*	*ycbV*
E200000238	1,760	0.895	*ECs0880*	-
E200003125	1,745	0.945	+:4432085~4432154	Predict_CGH_oligo
E100005076	1,780	0.139	*ECs1084*	-
E100000283	4,001	2,244	*yagX*	CDS
E100000778	1,782	1,035	*b0802*	*ybiJ*
E200001810	1,591	0.933	*b2148*	*mglC*
E100004983	1,307	0.123	*ECs1292*	-
E200002372	2,434	0.237	*c3786*	-
E100004695	2,561	0.27	+:690199~690268	-
E100000832	1,145	0.127	*b0856*	*potH*
E100002503	1,243	0.788	*b2552*	*hmp*
E200001300	2,155	1,397	*b1166*	*ymgB*
E100002199	1,433	0.18	*b2241*	*glpA*
E100001280	2,467	1,067	*b1309*	*ycjM*
E100001956	1,482	0.189	*b2861 | b1997 | b1403 | b4272 | b0360 | b4579 | b3044*	*insC-4 | insC-3 | insC-2 | insC-6 | insC-1 | yaiX | insC-5*
E100003468	1,220	0.818	*b3546*	*eptB*
E200001963	1,157	0.513	*c3032*	*yfgJ*

Table 20: Significant down regulated genes in IHE3034 compared to *matA* mutant at 20 °C. p≤ 0.05
sd: standard deviation

ID arrays	Mean (Stats)	SD (Stats)	Gene name	
E100005414	-1,136	0.283	*ECs2161*	-
E100001474	-2,420	0.041	*b1503*	*ydeR*
E100004207	-3,805	0.146	*b4320*	*fimH*
E100002437	-1,009	0.435	*b2486*	*hyfF*
E100002375	-1,095	0.501	*b2424*	*cysU*
E100000319	-1,064	0.487	*b0327*	*yahM*
E100003253	-2,267	0.16	*b3330*	*gspI*
E100005751	-1,211	0.088	*ECs4382*	-
E100002499	-2,671	0.197	*b2548*	*yphF*
E100003942	-2,044	0.155	*Z5648*	*yjbO*
E100004617	-1,044	0.563	*ECs0280*	-
E100003831	-1,213	0.658	*b3927*	*glpF*
E200000374	-1,622	0.903	*b2421*	*cysM*
E200002144	-1,122	0.099	*c3396*	-
E100002372	-1,469	0.843	*b2421*	*cysM*
E100004592	-1,862	0.173	*ECs0224*	-
E200001769	-2,506	0.259	*c2607*	-
E200000377	-1,461	0.892	*ECs3296*	-
E100002376	-1,035	0.408	*b2425*	*cysP*
E200002115	-1,187	0.13	*c3340*	-

Table 21: Avian top up regulated genes in BEN374 at 41 °C versus 37 °C. p≤ 0.05
sd: standard deviation

Reporter Name	SignificanceTest1535 :P :: isolation origin: avian Dataset	M1_mean (log2)	M1_sd
ykgE	0.00543993	1.26974924	0.35086892
araC	0.0047973	1.54955991	0.40986838
betI	6.58E-05	1.25933291	0.07818977
yedU	2.70E-05	3.97352485	0.1832899
cysJ	0.00888352	2.59508298	0.85196114
cysH	0.04874303	1.71133578	1.06435617
hdeB	0.00432877	1.04748358	0.26736674
cysD	0.00638976	3.68538456	1.07727588
hdhA	0.00019981	1.56809008	0.14120136
cysA	0.01873467	2.18591424	0.93977641
ykgF	0.00752802	1.07172367	0.33184566
katE	0.00050232	1.02019865	0.12517021
b0834	0.00039605	1.10588983	0.12526557
cysK	0.00885973	2.15558396	0.70700166
cysW	0.02311901	1.37360156	0.63866395
cysI	0.01922881	2.87463572	1.2478343
cysN	0.02931913	1.84116217	0.93684437
ybiI	0.02354102	1.17887444	0.55186429
slp	0.00089129	1.16061294	0.17275505
msyB	0.00078219	1.08581853	0.15465298
PAI I_*ORF55*	0.00057021	1.6095091	0.20607919
deoA	0.00096356	1.28919875	0.19701739
cdtB	0.00034247	1.30049663	0.14029228
ME_EO_28C_*orf8_1*	0.00034578	1.23028144	0.13314663
ME_EO_28C_*orf7_1*	0.00071355	1.21381028	0.16760954
cdtC	0.00048255	1.27121131	0.15387641
ME_EO_28C_*rorf1_1*	0.00153983	1.04108357	0.18648747
deoC	0.00061479	1.32109817	0.17349344
cdtA	0.00141583	1.18962525	0.20710662
ME_EO_28C_*orf9_1*	0.00059416	1.15523527	0.14997762
betB	0.00036858	1.25641374	0.13892192
galT	0.00185744	1.19844404	0.22881511
b0833	0.00212187	1.01613398	0.20301395

Table 22: Avian top down regulated genes in BEN374 at 41 °C versus 37 °C. p≤ 0.05
sd: standard deviation

Reporter Name	SignificanceTest1535 :P :: isolation origin: avian Dataset	M1_mean (log2)	M1_sd
flgB	0.00023256	-2.01281786	0.19070144
flgF	0.0002785	-2.22206194	0.22364108
map	0.00016849	-1.01514352	0.08633757
cheB	7.53E-05	-3.7688382	0.24482141
fliL	5.64E-05	-2.26110959	0.1334074
cheA	0.00011989	-2.70961973	0.20564986
fliZ	0.00010914	-2.72955985	0.20075362
fliO	0.00029024	-2.40786427	0.24572189
fliC	0.00035036	-3.48807051	0.37916578
ydbU	0.00083735	-1.33476107	0.19453245
b1409	0.00034631	-1.07289447	0.11617281
fliH	0.00070188	-1.4269189	0.19594497
tap	0.00166095	-1.19696049	0.21999943
fliJ	0.00074401	-1.00858096	0.14124773
tar	0.00018122	-2.94120338	0.25632243
flhE	4.45E-06	-1.86979853	0.04726676
flhB	0.00082638	-1.97015526	0.28586187
flgK	6.87E-06	-2.94177159	0.08595024
flgL	6.69E-06	-1.95864195	0.05672199
csgC	0.00068452	-1.81089638	0.24658255
flgA	4.25E-05	-1.3646321	0.07323861
flgH	0.00019526	-2.35571882	0.21049316
yhjB	0.00083492	-1.44741667	0.21074456
yhjH	0.00062025	-3.47491202	0.45770529
yihW	0.00211656	-1.8436122	0.36802136
yhcI	0.04411655	-1.75815417	1.05019683
yhcH	0.03507661	-1.69380326	0.92384214
nanA	0.01985392	-1.8041949	0.79251664
nlp	0.00038377	-1.35014273	0.15132275
ycgR	0.00169024	-2.35119764	0.43472281
cheZ	0.0013391	-3.01210863	0.51457121
fliD	0.00241675	-3.59441125	0.75077439
aer	0.00150244	-1.69937997	0.30187626
b1773	0.00511914	-1.19631243	0.32365422
malE	0.01985466	-1.33125959	0.58478154
flgN	0.00016894	-2.87923658	0.24509403
flgC	0.00020136	-2.23760725	0.20201177
flgE	0.00036699	-2.51492268	0.27767125
fhuF	0.00998924	-1.04523021	0.35776244

tsr	0.00157304	-3.60524677	0.65050185
ynbD	0.00048208	-1.7430733	0.21092353
cheY	2.47E-05	-3.88065084	0.173739
fliG	0.00032325	-2.07362285	0.21940039
fliK	0.00324853	-1.84314649	0.42608528
cheW	0.00022486	-1.68610959	0.15795702
fliM	4.57E-05	-1.42290784	0.07820919
fliN	0.0005922	-1.71259429	0.22208988
fliA	0.00021158	-2.42582685	0.22266396
fliP	0.02156804	-1.36687341	0.61921823
flgJ	0.00054507	-1.74820157	0.22046687
b1742	0.00270855	-1.13293905	0.24605796
ompF	0.00722695	-1.34475013	0.41044479
fliS	0.0033748	-1.79265021	0.41988537
cheR	0.00080308	-1.2037265	0.17297841
motB	5.09E-05	-2.62965712	0.14985877
flhA	0.00017495	-1.32425005	0.11405464
b1936	0.00056956	-1.98718986	0.25433917
ycgC	0.00849791	-1.04375095	0.33731571
b1760	0.00107619	-1.15213419	0.18277749
flgD	0.00043529	-2.53682635	0.29661784
flgG	0.00032966	-2.53367721	0.26984862
flgI	0.00012266	-2.08904817	0.15976741

Table 23: Human top down regulated genes in IHE3034 at 41 °C versus 37 °C. p≤ 0.05
sd: standard deviation

Reporter Name	SignificanceTest1535 :P :: isolation origin: human Dataset:	M1_mean	M1_sd
fes	0.00386284563881326	-1.15266614029998	0.428977845155756
flgB	0.00165791005509875	-1.91183705801925	0.567038164346919
flgF	0.000287636997653689	-2.17397106215668	0.409235730353247
cheB	0.000821598119843325	-2.3429717961473	0.578086171209726
fliL	0.000307607272573077	-2.26425342270238	0.433618014797015
cheA	0.000284861975609235	-1.32463484526167	0.24873601831891
fliZ	0.000780512250565772	-2.76935743864626	0.674237435320565
fliO	0.000243635467993889	-2.5939092943516	0.467995444443196
fliC	8.34672521255049e-05	-2.78646540843283	0.382907554248462
b1966	0.0011976961046789	-1.31821748074826	0.358886788469632
fepA	0.00343193512200591	-1.37306500084017	0.494819591040449
b1409	0.00129111920298342	-1.0197049508122	0.283144298498221
fliH	0.00140447644736106	-1.53154403226546	0.434786747565662
fliJ	0.000149579616436901	-1.07329524529617	0.171011114336878
tar	0.000766981686006271	-1.85626990631802	0.449885541079533
flhE	0.000375431684861607	-2.17737148514964	0.438836915269972
flhB	0.00035559479731418	-2.32258038953752	0.461626484711428
fliQ	7.18424993379995e-06	-1.29324159066566	0.0958330880869793
flgK	0.000613086519576464	-2.56135347528802	0.58576119257408
flgL	0.00163360463819056	-1.70623880018184	0.504086259054211
csgC	0.00539638291472146	-1.8625949712369	0.759929222247958
flgA	0.000907843569342958	-1.27759338306054	0.323522706616445
flgH	0.000404800575931716	-2.64289857057522	0.543067146592859
yhjB	0.00336033410467438	-1.01436002570629	0.363466312445492
yhjH	0.000945507005267344	-2.52742890540931	0.646835672963669
argT	0.0211219508414274	-1.17853621945433	0.715252597945729
nanT	0.0236263762774541	-1.22940835681496	0.772616122002189
yihW	0.0269574354827996	-1.08362064893766	0.709984778478855
fliF	0.000883922212047538	-1.20917012525715	0.304073075829504
nanA	0.0212245498255773	-1.16716976740421	0.709417081144638
nlp	5.38360606041522e-05	-1.45834142768851	0.179370965038798
oppD	0.00592653412209246	-1.01352954923035	0.424429545063899
ycgR	0.0149809176055956	-1.05268856926527	0.575587962125603
cheZ	0.000774330247263263	-1.91997457626174	0.466479006177085
fliD	0.000245758902350417	-3.14395078969126	0.568493092461791
ompC	0.000771816420381955	-1.60642288303423	0.389968612456984
ilvE	0.0193207355291711	-1.00771834469349	0.595034788187895
aer	0.000313845527328577	-1.78093530500525	0.342818017460301
yhaO	3.19243761481044e-05	-1.06051894981428	0.114332929074428
yeeY	0.0195227757218422	-1.14631235312373	0.679032405978833

proX	0.00523348380164017	-1.16043885611988	0.469449233522207
cstA	0.0321119873115126	-1.20179779833286	0.833139514211038
b0805	0.00123427396838618	-1.55897805360676	0.427797382562192
flgN	0.000375328402652755	-1.92149857315448	0.387239820519335
flgC	0.00118877257829624	-2.05595890698037	0.55864139997083
flgE	0.00126578652649858	-2.55235681236279	0.705040437119779
b2862	0.00647610328459191	-1.02491540129356	0.439966404129324
yjiY	0.0378351616725445	-1.50185173943065	1.09918639158887
tsx	0.020406266348883	-1.00639965516503	0.604318139550193
cspA	0.0250851851670845	-1.33665228715835	0.855997014867116
fhuF	0.00189758645581629	-1.02602119317015	0.315399631728912
tsr	0.0137824468905127	-2.13600252538727	1.13932673191206
cspE	0.0417738493782889	-1.0758194098531	0.814162981296411
ilvC	0.00317505182314715	-1.00371315378675	0.354169404906301
IHE3034_1_*CDS89_0_0*	0.0379643428356921	-1.05514668224678	0.773131167390603
ynbD	0.00274763253405157	-1.59183245521146	0.540209071881534
dgoA	0.0259397558264108	-1.4510698966484	0.939174554840198
cheY	0.000170869292270854	-3.29893698876288	0.543725101568598
b2809	0.00783762772373267	-1.51349240492987	0.685592747607731
fliG	0.000258616096326294	-2.39018222733125	0.437863663529782
fliK	0.000127552237935764	-2.02677253555812	0.31012269389102
fliM	0.000224477729746573	-1.57496213329067	0.278276645228968
fliY	0.00340502298195746	-1.15196255182291	0.414253775930393
fliN	0.000235132533760962	-2.05478787360906	0.367378898219459
fliA	0.000405730780830484	-2.60132362482032	0.534839677162428
fliP	6.43580411730899e-05	-2.13762729404731	0.275049934821335
flgJ	0.000319625082571068	-2.04796824504736	0.396067247810851
ompF	0.0152214392620137	-1.58090888802837	0.868529473776259
fliS	0.000148385595209592	-1.23772935732151	0.196809471505364
motB	0.00186180345915739	-1.26890930621845	0.388096865688319
flhA	4.25604196408359e-05	-2.17447880299794	0.252051301674394
b1936	0.00500213080767171	-2.14389728033412	0.856526245729641
yagU	0.0462922159546442	-1.01675962204753	0.797116147299299
flgD	0.00146376910448892	-2.46859727333852	0.708481470900605
flgG	0.000450228221522131	-2.51542862958143	0.531209222902688
flgI	0.000300209244571578	-2.31780826989561	0.441115854179044

Table 24: Human top up regulated genes in IHE3034 at 41 °C versus 37 °C. p≤ 0.05
sd: standard deviation

Reporter Name	SignificanceTest1535 :P :: isolation origin: human Dataset:	M1_mean	M1_sd
codB	0.01173818	1.40769138	0.71622353
b1498	0.00608196	1.55551794	0.65611535
yedU	3.26E-06	4.2714317	0.25956227
b1171	0.00402307	1.20381341	0.45301183
hdhA	0.00083504	1.74970674	0.4335345
yfeA	0.00065681	1.1317387	0.26347573
b0834	0.00089373	1.21187865	0.30563169
yggB	0.00097419	1.11584155	0.28780917
PAI I_*ORF55*	0.00028539	1.05891309	0.19893336
pyrI	0.01987001	1.46992707	0.87545328
pyrL	0.04121575	1.48148788	1.11604763
purK	0.03273331	1.09608159	0.76463036
ME_EO_ 28C_*rorf1_1*	6.94E-06	1.18153603	0.08680604
b1497	0.0034056	1.27501449	0.45852511
napD	0.00089112	1.18312757	0.29815342

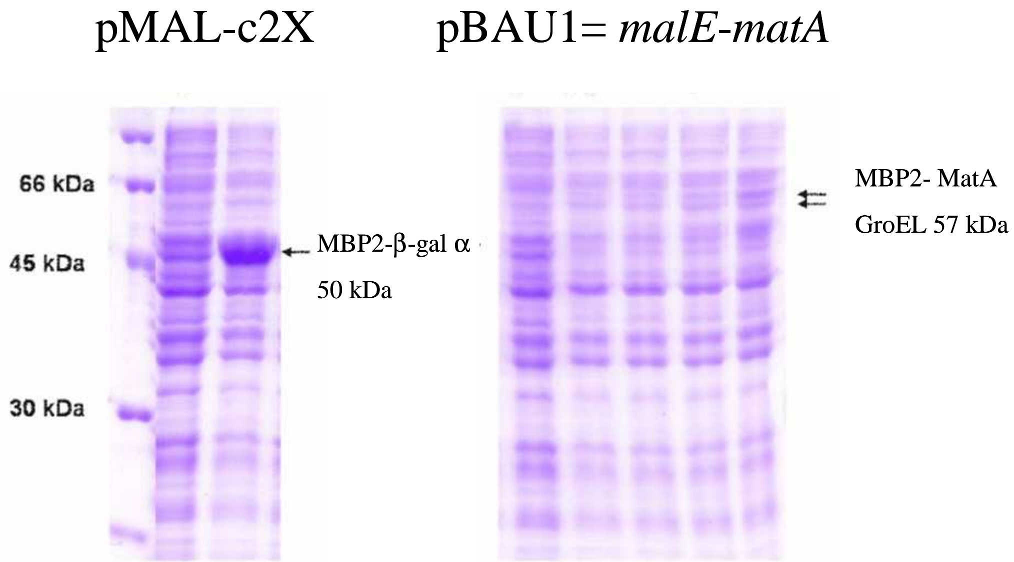

Figure 40: Analysis of protein expression of the protein fusion MalE-MatA (pBAU1). The expression was done in *Escherichia coli* BL21, at 37°C and in LB medium, following the manufacturer protocol (IPTG induction) and checked on SDS-PAGE, 12%.

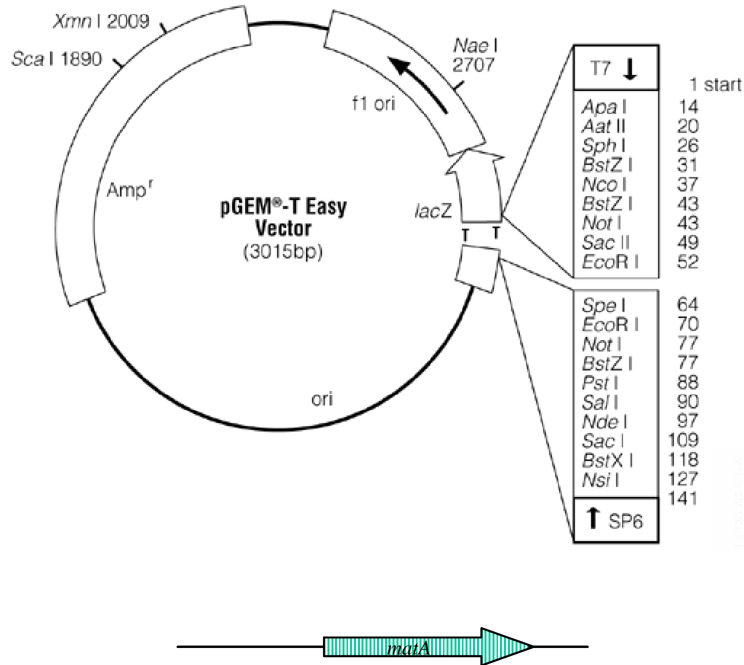

Figure 41: Cloning of *matA* and its promoter region of IHE3034. The cloning procedure was done following the manufacturer protocol, after amplification of *matA* and it promoter from the strain IHE3034 by the DAP polymerase, using the following primers, matA left 15 feb 2005 and intergenique matA reverse 8 february 2005. In PGEM-T Easy, the product was check by sequencing with the primer T7.

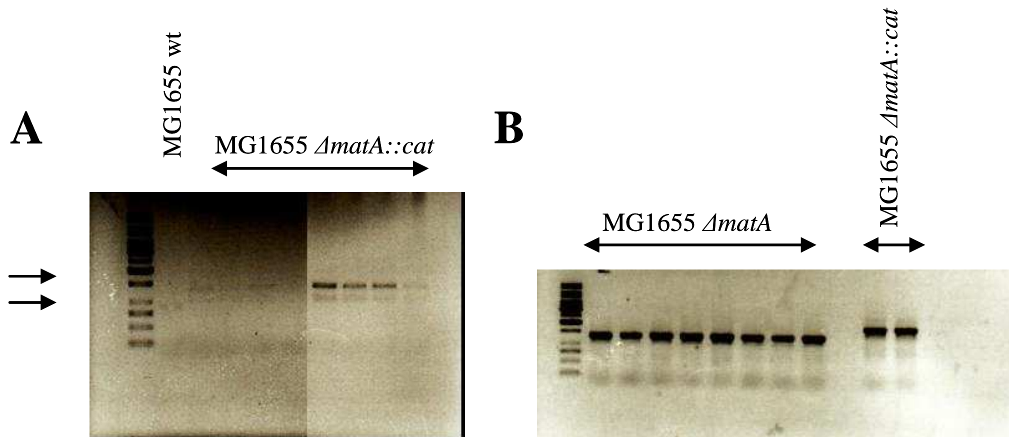

Figure 42: Construction of MG1655 *ΔmatA*. (A) Verification of matA deletion by λ red recombination and insertion of chloramphenicol resistance (*cat*) gene. PCR amplification using primer matA left and integenic complete right ctrl: wildtype strain before recombination. (B) Verification of *cat* gene deletionin MG1655. The antibiotic marker could be removed with the help of the FLP recombinase (encoded on plasmid pCP20), which mediates recombination between the two FRT sites flanking the antibiotic cassette, thus leaving behind a complete deletion of the open reading frame.

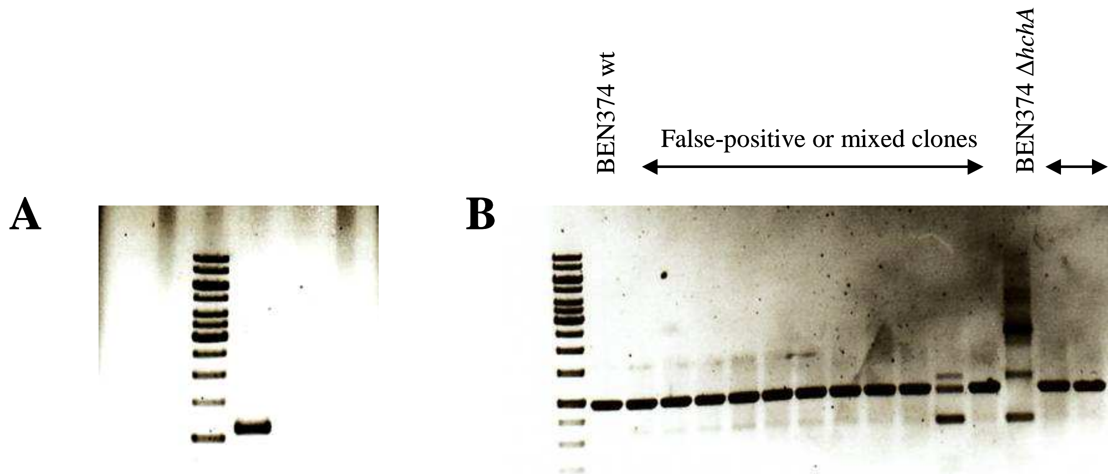

Figure 43: Construction of BEN374 *ΔhchA:: cat*. (A) PCR amplification of *cat* gene on pKD3. The construction of the mutants was performed using linear DNA for recombination, as described by Wanner and Datsenko (2000). This method relies on the replacement of a chromosomal sequence with an antibiotic marker that is generated by PCR using primers with homology extensions to the flanking regions of the target sequence (see method section 4.1.13). (B) PCR verification using primers binding outside the coding sequences of *hchA* (coding for YedU).

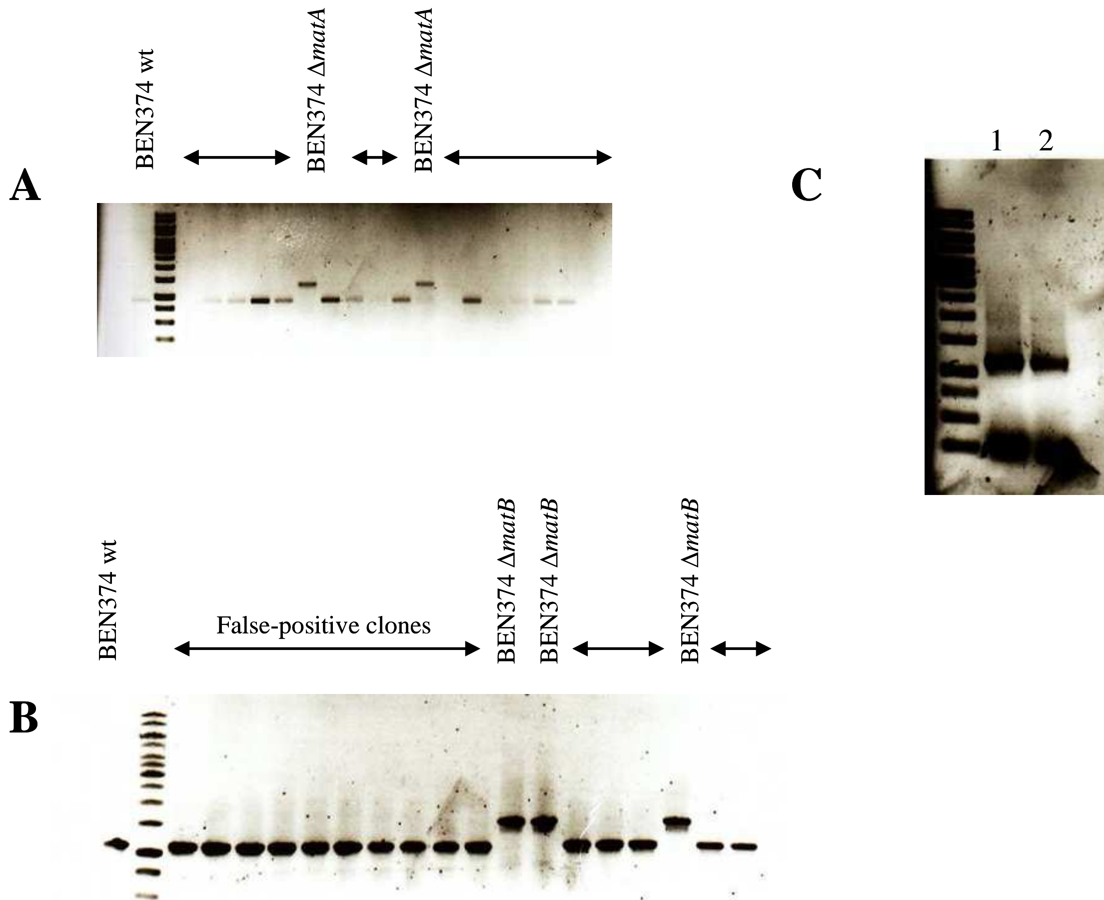

Figure 44: Construction of BEN374 *ΔmatA:: cat* and of BEN374 *ΔmatB:: cat*. (A) Construction of BEN374 *ΔmatA:: cat*. PCR verification using primers binding outside the coding sequences of *matA*. The arrows represent the false-positive clones. (B) Construction of BEN374 *ΔmatB:: cat*. PCR verification using primers binding outside the coding sequences of *matB*. (C) PCR amplification of *cat* gene on pKD3. The construction of the mutants was performed using linear DNA for recombination, as described by Wanner and Datsenko (2000). This method relies on the replacement of a chromosomal sequence with an antibiotic marker that is generated by PCR using primers with homology extensions to the flanking regions of the target sequence (see method section 4.1.13). 1: homology extensions to the flanking regions of *matA*; 2: homology extensions to the flanking regions of *matB*.

8.4 FliC expression analysis in IHE3034 and its isogenic mutant IHE3034 *ΔmatA*

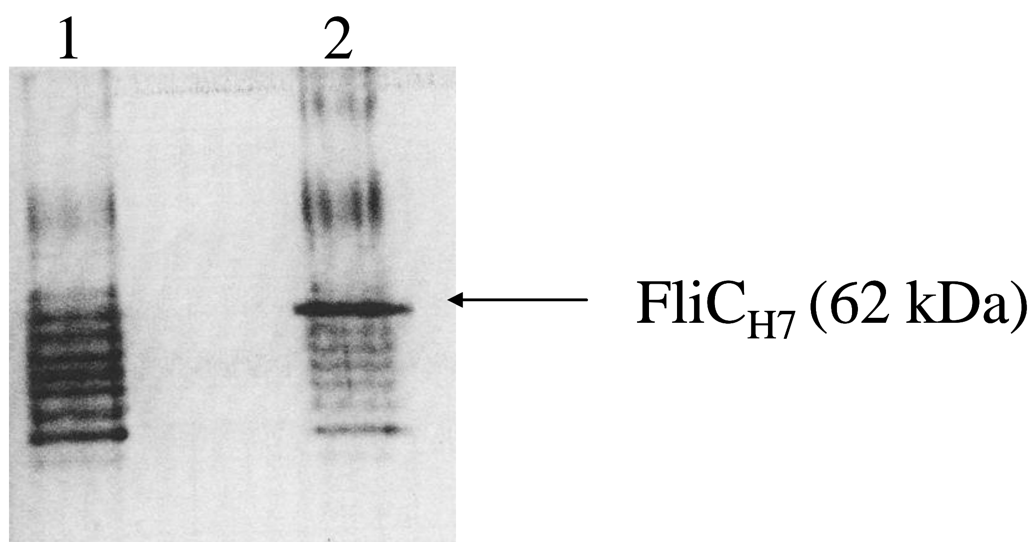

Figure 45: Analysis of FliC expression during the exponential phases at 20 °C and in LB medium. The immunoblot was performed on a whole-cell protein extract of IHE3034 and its isogenic mutant IHE3034 *ΔmatA*. Culture was performed in LB medium, at 20 °C, 200 rpm and for an OD_{600} 0.6. Lane 1: *E. coli* IHE3034; lane 2: *E. coli* IHE3034 *ΔmatA*. An overexpression of the FliC protein is proved in the mutant strain. It confirms the data known about the establishment of biofilm structure which needs a decrease of the motility when the fimbriae responsible of the biofilm, Mat fimbriae in our conditions, are expressed.

8.4 Abbreviations

µg = microgram
µl = microliter
µM = micromolar
µm = micrometer
A = adenine
a.a. = amino acid
AFM = atomic force microscope
Ap = Ampicillin
APS = ammonium persulfate
BLAST = Basic Local Alignment Search Tool
bp = base pairs
ß-ME = beta-mercaptoethanol
°**C =** grad celsius
C = cytosine
cAMP = cyclic adenosine monophosphate
CAS = chrome azurole S
cat = chloramphenicol acetyltransferase
CDS = coding sequence
CFU = colony forming unit
Cm = chloramphenicol
cm = centimeter
dATP = desoxyadenosin-5'-triphosphate
dCTP = desoxycytosin-5'-triphosphate
dGTP = desoxyguanosin-5'-triphosphate
dH2O = distilled water
dNTP = desoxynucleotide
dTTP = desoxythymidin-5'-triphosphate
DEPC = diethyl pyrocarbinate
DNA = desoxyribonucleic acid
DNase = Desoxyribonuclease
EAEC = enteroaggregative *E. coli*
EDTA = Ethylendiamintertraacetat
EHEC = enterohemorrhagic *E. coli*
EIEC = enteroinvasive *E. coli*
EPEC = enteropathogenic *E. coli*
EPS = extracellulat polymeric substance
et al. = et altera (and others)
ETEC = enterotoxigenic *E. coli*
EtOH = ethanol
ExPEC = extraintestinal pathogenic *E. coli*
Fig. = figure
Fis = factor for inversion stimulation
FPLC = Fast protein liquid chromatography
FRT = Flp recognition target

g = gram
G = guanine
GEI = genomic island
h = hour
HDTMA = hexadecyl-trimethyl-ammonium bromide
H-NS = histone-like nucleoid structuring protein
HU = heat-unstable nucleoid protein
IciA = inhibitor of chromosome initiation A
i.e. = id est (this means)
IPEC = intestinal pathogenic *E. coli*
kb = kilo bases
kDa = kilo Dalton
Km = Kanamycin
l = liter
LB = lysogeny broth
M = molar
mA = milliampere
mg = milligram
min = minute
ml = milliliter
mM = millimolar
mm = millimeter
MOPS =
NBM = newborn meningitis
ng = nanogramm
NMR = nuclear magnetic resonance
nt = nucleotides
OD = optical density
oligo = oligonucleotide
ON = overnight
ONPG = o-Nitrophenyl-ß-galactopyranoside
ORF = open reading frame
PAI = pathogenicity island
PBS = Phosphate buffered saline
PCR = polymerase chain reaction
PIPES = 1,4-piperazine-diethane-sulfonic acid
RNA = ribonucleic acid
RNase = Ribonuclease
Rob = replication origin binding protein
rpm = rounds per minute
RT = reverse transcription
SD = standard deviation
SDS = sodium dodecyl sulfate
sec = second
Spec = Spectinomycin
SSC = standard saline citrate

T = thymine
TAE = Tris-acetate-EDTA
TBS = Tris-buffered saline
TEMED = N,N,N`,N`-tetramethyldiamin
Tet = Tetracyclin
Tris = Trishydroxylmethylaminomethan
U = enzyme unit (1 U=1 µmol substrate × min-1)
UPEC = uropathogenic *Escherichia coli*
UTI = Urinary tract infection
V = Volt
v/v = volume/volume
wt = wild type
w/v = weight/volume
X-gal = 5-bromo-4-chloro-3-indolyl-β-glucoside

8.5 Curriculum vitae

PHILIPPE BAUCHART
Date and place of birth: 29.12.79, in Toulouse (France)

WORK EXPERIENCE

Jan 2004 - Jun 2008 : **Institute for Molecular Infection Biology Würzburg, Germany**
PhD fellow from the Bavarian research foundation (Supervisor: Prof. J. Hacker)

- Lead a research project in the field of *Escherichia coli* strains involved in extraintestinal infection and the characterisation of new virulence factors
- Managed a successful collaboration with 2 laboratories from Helsinki (Finland) and Tours (France) in the context of an European project

Feb - Jul 2003 : **National Veterinary school of Toulouse, France**
DEA internship (Supervisor: Prof. E. Oswald)
"Development and optimisation of a new reporter system to study the translocation of a new effector molecule of the type III secretion system in *Escherichia coli* pathogen"

Sep 2002 - Jan 2003 : **National Institute of Applied Sciences (INSA), Toulouse, France**
DEA Internship (Supervisor: Dr. A. Marty)

"Characterisation and molecular evolution of lipase LIP2 from *Yarrowia lipolytica*"

Jul 2001 - Sep 2001 : **Institute Claudius Régaud, Toulouse, France**
Voluntary internship (Supervisor: Dr. D. Lautier)

"Oncogenic expression of NPM-ALK: Sensibility test vis-à-vis of genotoxic reagents and involvement of the DNA repair mechanisms"

EDUCATION

2004-2008: PhD position at the Institute for Molecular Infection Biology, Würzburg, Germany. Subject: Evaluation of the zoonotic risk of *Escherichia coli* strains involved in extraintestinal infection of humans and avians

2002-2003: DEA (diploma after 5 years of higher education) in Biology-Health-Biotechnologies: Microbiology: Physiology and Molecular genetics. With honors. (Paul Sabatier University, Toulouse, France)

2000-2002: Master's degree in Cellular Biology, direction Microbiology. With honors, Major. (Paul Sabatier University, Toulouse, France)

1999-2000: Two-year university degree in cellular biology. (Paul Sabatier University, Toulouse, France)

1997-1999: First cycle of medical studies. (Paul Sabatier University, Toulouse, France)

1997: Baccalauréat S SVT, French equivalent to the High School Diploma. With Honors.

LAB SKILLS

Molecular Biology: DNA/ RNA extraction/ purification, cloning, PCR, RACE-PCR, Real –time RT-PCR, primer extension, northern blot, Southern blot, genome (sequencing and SNP analysis) and transcriptome (microarrays and statistics) analysis, cloning, directed mutagenesis, gene fusion, pulse field gel electrophoresis (PFGE).

Biochemistry: SDS PAGE**, w**estern blot, protein purification, construction and validation of reporter systems**,** enantioselectivity studies**,** ELISA, enzymatic essays, cytotoxic test.

Cellular biology: cell culture (eukaryote and prokaryote), host-pathogen interaction, fermentation (2 liters bioreactor), fluorescence microscopy, mutagenesis test, biofilm formation test.

Bioinformatic: Vector NTI, Modelling software on Silicon Graphics O2 R.10000 workstation (Insight II, Builder, Biopolymer, Discover), microarray analysis software (GenePix Pro6.0, EMMA software 2.4) and statistical analysis software (R software, Acuity software), analysis and comparison software for genomes and sequences (Artemis, blast, clustlaw, BioEdit). Good computer skills in MS Office, EndNote.

ENGLISH WORKSHOPS

2007 : “Oral presentation”, ICCON, Dr. A. Roos (Würzburg, Germany)

2006 : “Drug target analysis; From genome to drug targets; Modelling and docking; Genome analysis” (Würzburg, Germany)

2006 : “Human and Microbial genomics: DNA Arrays applied to Human Pathogens and Diseases”, FEBS formation (Athens, Greece)

2006 : “Effective Scientific writing” and “Poster Design and Poster Presentation”, Bioscript International, Dr. R. Willmott (Würzburg, Germany)

2005 : “Microarray Data Analysis with EMMA 2.0” (Bielefied, Germany)

FOREIGN LANGUAGES

- English: fluent in spoken and written
- German: basics (4 years in Germany)
- Spanish: basics

PUBLICATION & POSTERS

U. Dobrindt, J. Zdziarski1, **P. Bauchart**, S. Reidl, E. Brzuszkiewicz , G. Gottschalk, C. Svanborg, and J. Hacker. Genome fluidity and its impact on virulence gene expression and evolution of extraintestinal pathogenic *E. coli*. Nova Acta Leopoldina NF 98(359):97-103.

M. Cancino, **P. Bauchart**, G. Sandoval, J.M. Nicaud, I. André, V. Dossat, A. Marty (2008). “A variant of *Yarrowia lipolytica* lipase with improved activity and enantioselectivity for resolution of 2-bromo-arylacetic acid esters”. Tetrahedron: Asymmetry, 19(13)1608-1612

Timo A. Lehti, **Philippe Bauchart**, Maini Kukkonen, Ulrich Dobrindt,
Timo K. Korhonen, and Benita Westerlund-Wikström (2009). Adaptation by promoter variation: Pathogroup-specific expression of fimbrial *mat* operon in *E. coli*. FEMS (Gothenberg, Sweden).

G. Rouquet, G. Porcheron, N. Chanteloup, C. Barra1, **P. Bauchart**, U. Dobrindt, and Philippe Gilot (2008). Frz, une région métabolique impliquée dans la régulation de gènes de virulence et d'adaptation à l'hôte des *Escherichia coli* à pathogénicité extra-intestinale. The XXIe Colloque Biotechnocentre (Seillac, France).

G. Rouquet, G. Porcheron, N. Chanteloup, C. Barra1, **P. Bauchart**, U. Dobrindt, and Philippe Gilot (2008). An Extra-intestinal Pathogenic *Escherichia coli* metabolic region involved in host adaptation and virulence genes regulation. The 7th Louis Pasteur Conference on Infectious Diseases (Paris, France).

P. Bauchart, U. Dobrindt, and J. Hacker (2007). "Analysis of the zoonotic risk of extraintestinal pathogenic *Escherichia coli*" EMBO-FEMS-LEOPOLDINA Symposium: "*Escherichia coli* - Facets of a versatile pathogen" (Kloster Banz, germany).

P. Bauchart, U. Dobrindt, and J. Hacker (2007) "Zoonotic risk of extraintestinal pathogenic *Escherichia coli*". FEBS Congress (Vienna, Austria)

P. Bauchart, U. Dobrindt, and J. Hacker (2006) "Analysis of the zoonotic risk of extraintestinal pathogenic *Escherichia coli*". VAAM (Jena, Germany)

P. Bauchart, D. Guieysse, L. Roncalli, C. Croux, J.M. Nicaud, P. Monsan and A. Marty (2003) "Enantioselectivity improvement of *Yarrowia lipolytica* lipase for resolution of 2-substituted carboxylic esters". BioTrans 2003 (Olomouc, Czech Republic)